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(54) Title: DRUG DELIVERY PRODUCT AND METHODS

(57) Abstract: The present invention provides a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a payload molecule and a payload trapping molecule. The invention further provides methods of making and methods of using the particulate delivery system.

DRUG DELIVERY PRODUCT AND METHODS

BACKGROUND OF THE INVENTION

Drug delivery systems are designed to provide a biocompatible reservoir of an active agent for the controlled release of the active agent dependent either on time, or on local conditions, such as pH. While macroscopic drug delivery systems such as transdermal patches, implantable osmotic pumps and implantable subcutaneous depots (e.g., NORPLANT™) have had some success, there has been continuing interest in microscopic drug delivery systems such as microcapsules, microparticles and liposomes.

Microcapsules and microspheres are usually powders consisting of spherical particles 2 millimeters or less in diameter, usually 500 microns or less in diameter. If the particles are less than 1 micron, they are often referred to as nanocapsules or nanospheres. A description of methods of making and using microspheres and microcapsules can be found, for example in U.S. Pat. No. 5,407,609. Microcapsules and microspheres can be distinguished from each other by whether the active agent is formed into a central core surrounded by an encapsulating structure, such as a polymeric membrane, or whether the active agent is dispersed throughout the particle; that is, the internal structure is a matrix of the agent and excipient, usually a polymeric excipient. The release of the active agent from a microcapsule is often regulated by the biodegradation of the matrix material, usually a biodegradable polymeric material such as either poly(DL-lactide) (DL-PL) or poly(DL-lactide-co-glycolide) (DL-PLG) as the polymeric excipient.

Liposomes can be considered microcapsules in which the active agent core is encompassed by a lipid membrane instead of a polymeric membrane. Liposomes are artificial lipid vesicles consisting of lipid layers, where the antigen may be encapsulated inside the aqueous compartment of the liposome, or associated with the antigen on the surface via surface-coupling techniques. Liposomes can be prepared easily and inexpensively on a large scale and under conditions that are mild to entrapped antigens. They do not induce immune responses to themselves, and are used in humans for parenterally administered drugs.

While the high surface area / volume ratio of microcapsules, microspheres and liposomes favor the release of the active agent, their small size provides challenges in manufacturing. A wide variety of methods to prepare microcapsules and microspheres are described in the literature, e.g., U.S. Pat. No.5,407,609. Several of these methods make use of emulsions to make microspheres, in particular to make microspheres less than 2 millimeters in diameter. To give a general example of such processes, one can dissolve a polymer in a suitable organic solvent (the polymer solvent), dissolve or disperse an agent in this polymer solution, disperse the resulting

polymer/agent mixture into an aqueous phase (the processing medium) to obtain an oil-in-water emulsion with oil microdroplets dispersed in the processing medium, and remove the solvent from the microdroplets to form microspheres. These processes can also be performed with water-in-oil emulsions and with double emulsions. The use of emulsion-based processes that follow this basic approach is described in several U.S. patents, such as U.S. Pat. Nos. 3,737,337, 3,891,570, 4,384,975, 4,389,330, and 4,652,441.

Alternatively, extracted yeast cell wall particles are readily available, biodegradable, substantially spherical particles about 2-4 μm in diameter. Preparation of extracted yeast cell wall particles is known in the art, and is described, for example in U.S. Pat. Nos. 4,992,540, 5,082,936, 5,028,703, 5,032,401, 5,322,841, 5,401,727, 5,504,079, 5,968,811, 6,444,448 B1, 6,476,003 B1, published U.S. applications 2003/0216346 A1, 2004/0014715 A1, and PCT published application WO 02/12348 A2. A form of extracted yeast cell wall particles, referred to as "whole glucan particles," have been suggested as delivery vehicles, but have been limited either to release by simple diffusion of active ingredient from the particle or release of an agent chemically crosslinked to the whole glucan particle by biodegradation of the particle matrix. See U.S. Pat. Nos.5,032,401 and 5,607,677.

Extracted yeast cell wall particles, primarily due to their beta-glucan content, are targeted to phagocytic cells, such as macrophages and cells of lymphoid tissue. The mucosal-associated lymphoid tissue (MALT) comprises all lymphoid cells in epithelia and in the lamina propria lying below the body's mucosal surfaces. The main sites of mucosal-associated lymphoid tissues are the gut-associated lymphoid tissues (GALT), and the bronchial-associated lymphoid tissues (BALT).

Another important component of the GI immune system is the M or microfold cell. M cells are a specific cell type in the intestinal epithelium over lymphoid follicles that endocytose a variety of protein and peptide antigens. Instead of digesting these proteins, M cells transport them into the underlying tissue, where they are taken up by local dendritic cells and macrophages.

M cells take up molecules and particles from the gut lumen by endocytosis or phagocytosis. This material is then transported through the interior of the cell in vesicles to the basal cell membrane, where it is released into the extracellular space. This process is known as transcytosis. At their basal surface, the cell membrane of M cells is extensively folded around underlying lymphocytes and antigen-presenting cells, which take up the transported material

released from the M cells and process it for antigen presentation.

A study has shown that transcytosis of yeast particles (3.4 +/- 0.8 micron in diameter) by M cells of the Peyer's patches takes less than 1 hour (Beier, R., & Gebert, A., Kinetics of particle uptake in the domes of Peyer's patches, Am J Physiol. 1998 Jul;275(1 Pt 1):G130-7). Without significant phagocytosis by intraepithelial macrophages, the yeast particles migrate down to and across the basal lamina within 2.5-4 hours, where they quickly get phagocytosed and transported out of the Peyer's patch domes. M cells found in human nasopharyngeal lymphoid tissue (tonsils and adenoids) have been shown to be involved in the sampling of viruses that cause respiratory infections. Studies of an *in vitro* M cells model have shown uptake of fluorescently labeled microspheres (Fluospheres, 0.2 μm) and chitosan microparticles (0.2 μm) van der Lubben I.M., et al., Transport of chitosan microparticles for mucosal vaccine delivery in a human intestinal M-cell model, J Drug Target, 2002 Sep;10(6):449-56. A lectin, *Ulex europaeus* agglutinin 1 (UEA1, specific for alpha-L-fucose residues) has been used to target either polystyrene microspheres (0.5 μm) or polymerized liposomes to M cells (0.2 μm) (Clark, M.A., et al., Targeting polymerised liposome vaccine carriers to intestinal M cells, Vaccine. 2001 Oct 12;20(1-2):208-17). *In vivo* studies in mice have reported that poly-D,L-lactic acid (PDLLA) microspheres or gelatin microspheres (GM) can be efficiently taken up by macrophages and M cells. (Nakase, H., et al., Biodegradable microspheres targeting mucosal immune-regulating cells: new approach for treatment of inflammatory bowel disease, J Gastroenterol. 2003 Mar;38 Suppl 15:59-62).

However, it has been reported that uptake of synthetic particulate delivery vehicles including poly(DL-lactide-co-glycolide) microparticles and liposomes is highly variable, and is determined by the physical properties of both particles and M cells. Clark, M.A., et al., Exploiting M cells for drug and vaccine delivery, Adv Drug Deliv Rev. 2001 Aug 23;50(1-2):81-106. The same study reported that delivery may be enhanced by coating the particles or liposomes with reagents including appropriate lectins, microbial adhesins and immunoglobulins which selectively bind to M cell surfaces. See also, Florence, A.T., The oral absorption of micro- and nanoparticulates: neither exceptional nor unusual, Pharm Res. 1997 Mar;14(3):259-66.

Pathogen pattern recognition receptors (PRRs) recognize common structural and molecular motifs present on microbial surfaces and contribute to induction of innate immune responses. Mannose receptors and beta-glucan receptors in part participate in the recognition of fungal pathogens. The mannose receptor (MR), a carbohydrate-binding receptor expressed on

subsets of macrophages, is considered one such PRR. Macrophages have receptors for both mannose and mannose-6-phosphate that can bind to and internalize molecules displaying these sugars. The molecules are internalized by endocytosis into a pre-lysosomal endosome. This internalization has been used to enhance entry of oligonucleotides into macrophages using bovine serum albumin modified with mannose-6-phosphate and linked to an oligodeoxynucleotide by a disulfide bridge to a modified 3' end; see Bonfils, E., et al., Nucl. Acids Res. 1992 20, 4621-4629. see E. Bonfils, C. Mendes, A. C. Roche, M. Monsigny and P. Midoux, Bioconj. Chem., 3, 277-284 (1992). Macrophages also express beta-glucan receptors, including CR3 (Ross, G.D., J.A. Cain, B.L. Myones, S.L. Newman, and P.J. Lachmann. 1987. Specificity of membrane complement receptor type three (CR_3) for β-glucans. *Complement Inflamm. 4:61*), dectin-1. (Brown, G.D. and S. Gordon. 2001. Immune recognition. A new receptor for β-glucans. *Nature 413:36.*), and lactosylceramide (Zimmerman JW, Lindermuth J, Fish PA, Palace GP, Stevenson TT, DeMong DE. A novel carbohydrate-glycosphinglipid interaction between a beta-(1-3)-glucan immunomodulator, PGG-glucan, and lactosylceramide of human leukocytes. J Biol Chem. 1998 Aug 21:273(34):22014-20.). The beta-glucan receptor, CR_3 is predominantly expressed on monocytes, neutrophils and NK cells, whereas dectin-1 is predominantly expressed on the surface of cells of the macrophages. Lactosylceramide is found at high levels in M cells. Microglia can also express a beta-glucan receptor (Muller, C.D., et al. Functional beta-glucan receptor expression by a microglial cell line, Res Immunol. 1994 May;145(4):267-75).

There is evidence for additive effects on phagocytosis of binding to both mannose and beta-glucan receptors. Giaimis et al. reported observations suggesting that phagocytosis of unopsonized heat-killed yeast (*S. cerevisiae*) by murine macrophage-like cell lines as well as murine peritoneal resident macrophages is mediated by both mannose and beta-glucan receptors. To achieve maximal phagocytosis of unopsonized heat-killed yeast, coexpression of both mannose and beta-glucan receptors is required (Giaimis, J., et al., Both mannose and beta-glucan receptors are involved in phagocytosis of unopsonized, heat-killed *Saccharomyces cerevisiae* by murine macrophages, J Leukoc Biol. 1993 Dec;54(6):564-71).

SUMMARY OF THE INVENTION

In a preferred embodiment, the present invention provides a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan and a payload trapping molecule. The particulate delivery system optionally, but typically, also includes a payload molecule, wherein the payload molecule and the payload trapping molecule are soluble in the same solvent system. In preferred embodiments, the solvent system comprises water. In other preferred embodiments, the solvent system consists essentially of water. The particulate delivery system of the present invention is useful for both *in vivo* and *in vitro* delivery of payload molecules to cells.

In particularly preferred embodiments, extracted yeast cell wall comprises less than 90 weight percent beta-glucan. In certain preferred embodiments, the extracted yeast cell wall comprises more than 50 weight percent chitin. In other preferred embodiments, the extracted yeast cell wall further comprises more than 30 weight percent mannan. In certain embodiments, the extracted yeast cell wall includes more than 1 weight percent protein.

In preferred embodiments, the payload molecule is selected from the group consisting of a polynucleotide, a peptide, a protein, a small organic active agent, a small inorganic active agent and a mixture thereof. In certain preferred embodiments, the payload molecule is a polynucleotide selected from the group consisting of an oligonucleotide, an antisense construct, a siRNA, an enzymatic RNA, a recombinant DNA construct, an expression vector, and a mixture thereof. In other preferred embodiments, the particulate delivery system of the present invention is useful for *in vivo* or *in vitro* delivery of payload molecules such as amino acids, peptides and proteins. The peptides can be signaling molecules such as hormones, neurotransmitters or neuromodulators, and can be the active fragments of larger molecules, such as receptors, enzymes or nucleic acid binding proteins. The proteins can be enzymes, structural proteins, signaling proteins or nucleic acid binding proteins, such as transcription factors.

In other preferred embodiments, the payload molecule is a small organic active agent, such as a therapeutic agent or a diagnostic agent. In particularly preferred embodiments, the small organic active agent is a sequence-specific DNA binding oligomer, more preferably an oligomer of heterocyclic polyamides that bind to the minor groove of double stranded DNA, such as those disclosed in U.S. Pat. No. 6.506,906 and in Dervan, P. Molecular Recognition of DNA by Small Molecules, Bioorganic & Medicinal Chemistry (2001) 9: 2215-2235, both of which are hereby incorporated by reference. In preferred embodiments, the oligomer has

monomeric subunits selected from the group consisting of N-methylimidazole carboxamide, N-methylpyrrole carboxamide, beta-alanine and dimethylaminopripylamide.

In other preferred embodiments, the particulate delivery system of the present invention includes inorganic active agents, e.g., gastrointestinal therapeutic agents such as aluminum hydroxide, calcium carbonate, magnesium carbonate, sodium carbonate and the like.

The choice of the payload trapping molecule can confer specific characteristics to the particulate delivery system. In general, the preferred payload trapping molecule is biocompatible and pharmaceutically acceptable. As noted above, the payload molecule and the payload trapping molecule are soluble in the same solvent system. Suitable payload trapping molecules include natural and synthetic polymers. In certain embodiments, the physical characteristics of the payload trapping molecule, such as agarose or polyacrylamide, provide useful advantages

Suitable polymers include polysaccharides. In preferred embodiments, the polysaccharide selected is from the group consisting of agarose, an alginate, a xanthan, a dextran, a chitosan, a galactomannan gum, a derivative thereof and a mixture thereof. In certain preferred embodiments, the polysaccharides have been derivatized to produce cationic or anionic characteristics at physiological pH.

In other embodiments, the payload trapping molecule is a charged molecule at physiological pH, such as a cationic polymer, an anionic polymer, a cationic detergent, an anionic detergent and a mixture thereof. Preferred cationic polymers include chitosan, poly-L-lysine and polyethylenimines (PEIs), including substantially linear polyethylenimines, such as JetPEI, a commercially available linear polyethylenimine cationic polymer transfection reagent (Qbiogene, Inc., CA). Other cationic polymer transfection reagents are also suitable, preferably CytoPure™, a proprietary, commercially available, water-soluble cationic polymer transfection reagent (Qbiogene, Inc., CA). In other preferred embodiments, suitable anionic polymers include alginates, dextrans and xanthans, including derivatized alginates, dextrans and xanthans. In further preferred embodiments, the payload trapping molecule is a cationic detergent such as hexadecyltrimethylammoniumbromide. In one preferred embodiment, a mixture of a cationic detergent, such as hexadecyltrimethylammoniumbromide, and a cationic polymer, such as a polyethylenimine, is used. In another preferred embodiment, a mixture of a cationic detergent, such as hexadecyltrimethylammoniumbromide, and a cationic polymer, such as chitosan or PEI, can be used.

While not being held to a single hypothesis, it is believed that, in addition to facilitating the retention of the payload by the yeast cell wall particles, a preferred payload trapping molecule serves to encourage the release of the payload molecule from the endosome of a phagocytic cell by acting as a detergent, by helping to swell the endosome osmotically, or by other effects.

In other preferred embodiments, the present invention provides methods of using the particulate delivery system. In certain preferred embodiments, the invention provides methods of delivering a payload molecule to a cell comprising the steps of providing a extracted yeast cell wall comprising beta-glucan, the yeast cell wall defining an internal space; contacting the extracted yeast cell wall with a payload molecule wherein the payload molecule becomes at least partially enclosed within the internal space; contacting the extracted yeast cell wall with a payload trapping molecule wherein the payload trapping molecule at least partially confines the payload molecule within the extracted yeast cell wall to form a particulate delivery system; and contacting a cell with the particulate delivery system. Preferably the method further includes the step of internalizing the particulate delivery system by the cell.

In other preferred embodiments, the invention provides methods of making a particulate delivery system comprising the steps of providing a extracted yeast cell wall comprising beta-glucan, the yeast cell wall defining an internal space; contacting the extracted yeast cell wall with a payload molecule wherein the payload molecule becomes associated with the extracted yeast cell wall; contacting the extracted yeast cell wall with a payload trapping molecule wherein the payload trapping molecule stabilizes the association of the payload molecule with the extracted yeast cell wall to form a particulate delivery system. In preferred embodiments, the method also includes the steps of washing and drying the particulate delivery system.

In other preferred embodiments, the present invention provides methods of exposing an individual to an antigen comprising the step of contacting a phagocytic cell of the individual with a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a payload trapping molecule and payload molecule, wherein the payload molecule is a polynucleotide comprising a control element operatively linked to an open reading frame encoding a peptide that can be controllably expressed in the cells of the individual. Preferably the encoded peptide is an antigenic peptide. In further preferred embodiments, the present invention provides methods of exposing an individual to an antigen comprising the step of contacting a phagocytic

cell of the individual with a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a payload trapping molecule and payload molecule, wherein the payload molecule is a antigenic molecule. A preferred antigenic molecule is a toxoid.

In preferred embodiments, the contacted cells are macrophages, but may also include any cell capable of yeast particle phagocytosis, including M cells of the Peyer's patches, monocytes, neutrophils, dendritic cells, Langerhans cells, Kupffer cells, alveolar phagocytes, peritoneal macrophages, milk macrophages, microglia, eosinophils, granulocytes, mesengial phagocytes, synovial A cells and other phagocytes. In preferred embodiments, the particulate delivery system is administered orally and absorbed via M cells of the Peyer's patches in the gut.

In preferred embodiments the polynucleotide is a recombinant DNA construct comprising a control element operatively linked to an open reading frame encoding a protein, e.g. an expression vector. The protein can be a structural protein, a protein having enzymatic activity, a membrane protein a DNA binding protein or a signaling protein. In certain preferred embodiments, the protein is an antigenic protein.

In certain preferred embodiments, the method further includes the step of the cell expressing the protein. The expressed protein can be retained intracellularly by the cell, incorporated in a membranous structure, such as the plasma membrane, or be secreted by the cell.

In other embodiments, more than one type of polynucleotide is enclosed within the particulate delivery system. In preferred embodiments, the polynucleotides provide the ability to express multiple gene products under control. In certain embodiments, at least one expressible gene product is a membrane protein, preferably a membrane receptor, most preferably a membrane-bound receptor for a signaling molecule. In some embodiments, at least one expressible gene product is a soluble protein, preferably a secreted protein, most preferably a signaling protein or peptide.

In other embodiments, the present invention provides a method of delivering a drug to a macrophage cell including the steps of providing a substantially spherical extracted yeast cell wall comprising beta-glucan, the yeast cell wall defining an internal space; contacting the extracted yeast cell wall with a drug wherein the drug is at least partially enclosed within the internal space; contacting the extracted yeast cell wall with a trapping molecule wherein the trapping molecule is at least partially enclosed within the internal space to form a particulate drug delivery system; and contacting a macrophage cell with the particulate drug delivery

system. Preferably, the method also includes the step of internalizing the particulate drug delivery system by the macrophage. In preferred embodiments, the method also includes the step of transporting the particulate drug delivery system by the macrophage. In particularly preferred embodiments, the macrophage delivers the particulate drug delivery system to a macrophage-attracting site, such as a site of infection, inflammatory reaction, hypoxia or hyperplasia. In certain preferred embodiments, the macrophage delivers the particulate drug delivery system to a tumor. In particularly preferred embodiments, the method includes the step of releasing the drug from the particulate drug delivery system, more preferably further including the step of releasing the drug into the extracellular space. In certain embodiments, the step of releasing the drug includes the steps of expressing a recombinant protein and secreting the protein into the extracellular space.

The present invention provides a method of immunizing an individual against a pathogen. The method comprises the step of contacting cells of said individual with a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a payload trapping molecule and a nucleic acid composition, thereby administering to the cells a nucleic acid molecule that comprises a nucleotide sequence that encodes a peptide which comprises at least an epitope identical to, or substantially similar to an epitope displayed on said pathogen as antigen, and said nucleotide sequence is operatively linked to regulatory sequences, wherein the nucleic acid molecule is capable of being expressed in the cells of the individual.

In another preferred embodiment, the present invention provides a method of producing immunity to a toxoid comprising the steps of providing a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a payload trapping molecule and a toxoid, contacting a phagocytic cell with the particulate delivery system and inducing phagocytosis of the particulate delivery system. The phagocytic cell can be one or more of macrophages, M cells of the Peyer's patches, monocytes, neutrophils, dendritic cells, Langerhans cells, Kupffer cells, alveolar phagocytes, peritoneal macrophages, milk macrophages, microglia, eosinophils, granulocytes, mesengial phagocytes, and synovial A cells.

The present invention provides methods of immunizing an individual against a hyperproliferative disease or an autoimmune disease. The methods comprise the step of contacting cells of said individual with a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a payload trapping molecule which includes a nucleic acid composition, thereby administering to the cells a nucleic acid molecule that comprises a

nucleotide sequence that encodes a peptide which comprises at least an epitope identical to, or substantially similar to an epitope displayed on a hyperproliferative disease-associated protein or an autoimmune disease-associated protein, respectively, and is operatively linked to regulatory sequences, wherein the nucleic acid molecule is capable of being expressed in the cells of the individual.

The present invention also provides methods of treating an individual suffering from an autoimmune disease comprising the steps of contacting cells said individual with a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a payload trapping molecule which includes a nucleic acid composition, thereby administering to the cells a nucleic acid molecule that comprises a nucleotide sequence that restores the activity of an absent, defective or inhibited gene, or that encodes a protein that produces a therapeutic effect in the individual, and is operatively linked to regulatory sequences; the nucleic acid molecule being capable of being expressed in said cells.

In further embodiments, the present invention provides a method of immunizing an individual against a hyperproliferative disease comprising the step of contacting cells of said individual with a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a payload trapping molecule and a payload molecule that is a polynucleotide comprising a control sequence operatively linked to an open reading frame encoding a peptide that comprises an epitope identical to, or substantially similar to, an epitope displayed on a hyperproliferative disease-associated protein, wherein encoded peptide is capable of being expressed in the cells of the individual. In other embodiments, the present invention provides a method of treating an individual suffering from a genetic disease comprising the step of contacting cells of said individual with a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a payload trapping molecule and a payload molecule that is a polynucleotide thereby administering to the cells a polynucleotide that comprises a nucleotide sequence that restores the activity of an absent, defective or inhibited gene. Preferably, the polynucleotide comprises a regulatory sequence operatively linked to an open reading frame encoding a protein that produces a therapeutic effect in the individual, the protein being capable of being expressed in said cells.

The present invention also relates to methods of treating an individual suffering from an autoimmune disease comprising the steps of contacting cells said individual with a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a payload

trapping molecule which includes a nucleic acid composition, thereby administering to the cells a nucleic acid molecule that comprises a nucleotide sequence that restores the function of an absent, defective or inhibited gene, or that encodes a protein that produces a therapeutic effect in the individual, and is operatively linked to regulatory sequences; the nucleic acid molecule being capable of being expressed in said cells.

Accordingly the present invention provides compositions and methods which prophylactically and/or therapeutically immunize an individual against a pathogen or abnormal, disease-related cell. The genetic material encodes a peptide or protein that shares at least an epitope with an immunogenic protein found on the pathogen or cells to be targeted. The genetic material is expressed by the individual's cells and serves as an immunogenic target against which an immune response is elicited. The resulting immune response is broad based: in addition to a humoral immune response, both arms of the cellular immune response are elicited. The methods of the present invention are useful for conferring prophylactic and therapeutic immunity. Thus, a method of immunizing includes both methods of protecting an individual from pathogen challenge, or occurrence or proliferation of specific cells, as well as methods of treating an individual suffering from pathogen infection, hyperproliferative disease or autoimmune disease. Thus, the present invention is useful to elicit broad immune responses against a target protein, i.e. proteins specifically associated with pathogens or the individual's own "abnormal" cells.

The present invention is also useful in combating hyperproliferative diseases and disorders such as cancer, by eliciting an immune response against a target protein that is specifically associated with the hyperproliferative cells. The present invention is further useful in combating autoimmune diseases and disorders by eliciting an immune response against a target protein that is specifically associated with cells involved in the autoimmune condition.

The present invention also provides pharmaceutical kits that comprise a container comprising a payload molecule selected from the group consisting of a nucleic acid composition, protein composition, small organic molecule and mixtures thereof, and a container comprising a yeast cell wall particle and a trapping molecule. Optionally, there is included in such kits excipients, carriers, preservatives and vehicles of the type described above with respect to pharmaceutical compositions. The term pharmaceutical kit is also intended to include multiple inoculants used in the methods of the present invention. Such kits include separate containers comprising different inoculants and transfer moieties. The pharmaceutical kits in accordance

with the present invention are also contemplated to include a set of inoculants used in the treatment and immunizing methods and/or therapeutic methods, as described above.

The compositions and methods of the present invention are useful in the fields of both human and veterinary medicine. Accordingly, the present invention relates to genetic immunization and therapeutic treatment of mammals, birds and fish. The methods of the present invention can be particularly useful for genetic immunization and therapeutic treatment of mammalian species including human, bovine, ovine, porcine, equine, canine and feline species.

The foregoing and other features and advantages of the particulate drug delivery system and methods will be apparent from the following more particular description of preferred embodiments of the system and method as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram 100 of a transverse section of a yeast cell wall, showing, from outside to inside, an outer fibrillar layer 110, an outer mannoprotein layer 120, a beta glucan layer 130, a beta glucan – chitin layer 140, an inner mannoprotein layer 150, the plasma membrane 160 and the cytoplasm 170.

Figure 2A is a diagram of the structure of a yeast cell wall particle; Figure 2B is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph showing staining of the mannan component of the yeast cell wall particles by concanavalin-A-FITC (con-A-fluorescein isothiocyanate, Sigma Chemical, St. Louis, MO) showing substantially completely stained yeast cell wall particles 210; Figure 2C is a diagram of the structure of a YGMP beta glucan-mannan particle, Figure 2D is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph showing patchy con-A-FITC staining of a YGMP beta glucan-mannan particle 220; Figure 2E is a diagram of the structure of a YGP beta glucan particle and Figure 2F is a reversed contrast (negative) grayscale image of a color fluorescence micrograph showing the absence of con-A-FITC staining.

Figure 3A is a reversed contrast (negative) grayscale image of a color light photomicrograph of cells exposed to YGP particles loaded with fluorescent labeled pIRES plasmid with PEI as the cationic trapping polymer and CTAB as a cationic detergent, indicating a cell 310 and Figure 3B is a reversed contrast (negative) grayscale image of a color fluorescence

photomicrograph of the same field of cells showing bright staining representing fluorescent YGP particles internalized by the same cell 310 indicated in Figure 3B.

Figure 4A is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph of cells, e.g., an indicated cell 410, exposed to fluorescent labeled YGP particles, Figure 4B is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph of cells, e.g., an indicated cell 420, exposed to YGP particles containing pIRES DNA, a cationic trapping polymer polyethylenimine (PEI) and cationic detergent hexadecyltrimethylammoniumbromide (also known as cetyltrimethylammonium bromide or CTAB) expressing GFP and Figure 4C is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph of cells, e.g., an indicated cell 430, exposed to YGP particles containing pIRES DNA, a cationic trapping polymer chitosan and cationic detergent CTAB expressing GFP.

Figure 5A is a reversed contrast (negative) grayscale image of a color combined light and fluorescence photomicrograph of cells, e.g., an indicated cell 510, exposed to fluorescent labeled YGP particles; Figure 5B is a graphic representation of the results of a fluorescence activated cell sorting (FACS) study showing a major peak 520 representing the distribution of signals from cells that have internalized fluorescent labeled YGP particles and a minor peak 530 representing the distribution of signals from cells without fluorescent labeled YGP particles; Figure 5C is a reversed contrast (negative) grayscale image of a color light photomicrograph of cells, e.g., an indicated cell 540, exposed to YGP particles containing fluorescent labeled DNA, a cationic trapping polymer PEI and cationic detergent CTAB; Figure 5D is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph of the same field of cells showing the same indicated cell 540, Figure 5E is a graphic representation of the results of a FACS study showing a major peak 610 representing the distribution of signals from cells that have internalized YGP particles with fluorescent DNA payload and a shoulder 620 representing the distribution of signals from cells without YGP particles; Figure 5F is a reversed contrast (negative) grayscale image of a color light photomicrograph of cells, e.g., an indicated cell 710, incubated with YGP particles containing fluorescent antisense RNA payload; Figure 5G is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph of the same field of cells showing the same indicated cell 710; Figure 5H is a reversed contrast (negative) grayscale image of a color light micrograph of cells, e.g., an indicated cell 810, incubated with YGP particles containing fluorescent labeled siRNA, PEI and CTAB and Figure

5I is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph of the same field of cells showing the same indicated cell 810 containing internalized YGP particles with fluorescent RNAi payload.

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

DETAILED DESCRIPTION OF THE INVENTION

In preferred embodiments, the invention provides a particulate delivery system comprising an extracted yeast cell wall particle and at least one payload trapping molecule. Preferably, the yeast cell wall particle is a 2-4 micrometer yeast cell wall ghost.

Payload Trapping Molecules

The payload trapping molecule is preferably a pharmaceutically acceptable excipient. The payload and trapping molecule are both soluble in the solvent system; the solvent system must be absorbed through the yeast cell particle carbohydrate matrix allowing the absorption of the payload and trapping polymer. The payload and trapping molecule are preferably water soluble. In preferred embodiments, the trapping molecule is biodegradable.

The mechanism of action of the trapping reaction with a given payload dictates the choice of payload trapping molecule. For electrostatic interactions a charged payload trapping molecule of opposite charge of the payload is required. For physical entrapment, the payload trapping molecule suitably participates in the formation of a matrix that reduces the diffusion of a payload. In other embodiments, the payload trapping molecule contributes a hydrophobic binding property that contributes to the retention of the payload. In further embodiments, the payload trapping molecule selectively binds to the payload, providing an affinity interaction that contributes to the retention of the payload.

In general, polyelectrolytes can be suitable payload trapping molecules. Several suitable polyelectrolytes are disclosed in U.S. Pat. No. 6,133,229. The polyelectrolyte may be a cationic or anionic polyelectrolyte. Amphoteric polyelectrolytes may also be employed. The cationic polyelectrolyte is preferably a polymer with cationic groups distributed along the molecular chain. The cationic groups, which in certain embodiments may include quaternary ammonium-derived moieties, may be disposed in side groups pendant from the chain or may be incorporated in it. Examples of cationic polyelectrolytes include: copolymers of vinyl pyrollidone and quaternary methyl methacrylate e.g., GAFQUAT®. series (755N, 734, HS-100) obtained from ISP; substituted polyacrylamides; polyethyleneimine, polypropyleneimine and substituted derivatives; polyamine homopolymers (GOLCHEM® CL118); polyamine co-polymers (e.g., condensates of epichlorohydrin and mono or dimethylamine); polydiallyl dimethyl ammonium chloride (polyDADMAC); substituted dextrans; modified guar gum (substituted with hydroxypropytrimonium chloride); substituted proteins (e.g., quaternary groups substituted on

soya protein and hydrolysed collagen); polyamino acids (e.g., polylysine); low molecular weight polyamino compounds (e.g., spermine and spermidine). Natural or artificial polymers may be employed. Cationic polyelectrolytes with MW 150 to 5,000,000, preferably 5000 to 500,000, more preferably 5000 to 100,000 may be employed. An amount of 0.01 to 10% is preferred, more preferably 0.1 to 2% w/v, especially 0.05 to 5%.

The anionic polyelectrolyte is preferably a polymer with anionic groups distributed along the molecular chain. The anionic groups, which may include carboxylate, sulfonate, sulphate or other negatively charged ionisable groupings, may be disposed upon groups pendant from the chain or bonded directly to the polymer backbone. Natural or artificial polymers may be employed.

Examples of anionic polyelectrolytes include: a copolymer of methyl vinyl ether and maleic anhydride, a copolymer of methyl vinyl ether and maleic acid, (Gantrez AN-series and S-series, respectively, International Specialty Products, Wayne, NJ); alginic acid and salts; carboxymethyl celluloses and salts; substituted polyacrylamides (eg substituted with carboxylic acid groups); polyacrylic acids and salts; polystyrene sulfonic acids and salts; dextran sulphates; substituted saccharides e.g., sucrose octosulfate; heparin. Anionic polyelectrolytes with MW of 150 to 5,000,000 may be used, preferably 5000 to 500,000, more preferably 5000 to 100,000. An amount of 0.01% to 10% is preferred especially 0.05 to 5% more especially 0.1 to 2% w/v.

Biological polymers, such as polysaccharides, are preferred trapping polymers. Preferably, the polymers are processed to an average molecular weight to less than 100,000 Daltons. The polymers are preferably derivatized to provide cationic or anionic characteristics. Suitable polysaccharides include chitosan (deacetylated chitin), alginates, dextrans, such as2-(diethylamino) ethyl ether dextran (DEAE-dextran) and dextran sulphate, xanthans, locust bean gums and guar gums.

Two general classes of cationic molecules are suitable for use as trapping molecules with negatively charged payloads such as polynucleotides: cationic polymers and cationic lipids.

A wide variety of cationic polymers have been shown to mediate *in vitro* transfection, ranging from proteins [such as histones (Fritz, J. D., et al, (1996) *Hum. Gene Ther.* **7**, 1395-1404) and high mobility group (HMG) proteins (Mistry, A. R.., et al. (1997) BioTechniques 22, 718-729)] and polypeptides [such as polylysine (Wu, G. Y. & Wu, C. H. (1987) J. Biol. Chem. 262, 4429-4432, Wagner, E., et al., (1991) Bioconjugate Chem. 2, 226-231, , short synthetic peptides (Gottschalk, S.,et al., (1996) Gene Ther. 3, 448-457; Wadhwa, M. S., et al., (1997)

Bioconjugate Chem. 8, 81-88), and helical amphiphilic peptides (Legendre, J. Y., et al., (1997) Bioconjugate Chem. 8, 57-63; Wyman, T. B., et al., (1997) Biochemistry 36, 3008-3017)] to synthetic polymers [such as polyethyleneimine (Boussif, O., et al., (1996) Gene Ther. 3, 1074-1080), cationic dendrimers (Tang, M. X., et al., (1996) Bioconjugate Chem. 7, 703-714; Haensler, J. et al., (1993) Bioconjugate Chem. 4, 372-379), and glucaramide polymers (Goldman, C. K., et al., (1997) Nat. Biotech. 15, 462-466)]. Other suitable cationic polymers include N-substituted glycine oligomers (peptoids) (Murphy, J.E., et al, A combinatorial approach to the discovery of efficient cationic peptoid reagents for gene delivery, Proc Natl Acad Sci. USA, 1998 95 (4)1517-1522), poly(2-methyl-acrylic acid 2-[(2-dimethylamino)-ethyl)-methyl-amino] -ethyl ester), abbreviated as pDAMA, and poly(2-dimethylamino ethyl)-methacrylate (pDMAEMA) (Funhoff, A.M., et al., 2004 Biomacromolecules, 5, 32-39).

Cationic lipids are also known in the art to be suitable for transfection. Felgner, P.Ll, et al., Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure.Proc Natl Acad Sci U S A. 1987 84(21):7413-7. Suitable cationic lipids include N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), [N,N,N′,N′-tetramethyl- N,N′-bis(2-hydroxyethyl)-2,3-di(oleoyloxy)-1,4-butanediammonium iodide] (Promega Madison, WI, USA), dioctadecylamidoglycyl spermine (Promega Madison, WI, USA), N-[1-(2,3-Dioleoyloxy)]-N,N,N-trimethylammonium propane methylsulfate (DOTAP), N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide (DMRIE), dimyristoleoyl phosphonomethyl trimethyl ammonium (DMPTA) (see Floch et al. 1997. Cationic phosphonolipids as non-viral vectors for DNA transfection in hematopoietic cell lines and CD34+ cells. Blood Cells, Molec.& Diseases 23: 69-87), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl), ammonium salt (Avanti Polar Lipids, Inc. Alabaster, AL, US), 1,2-dioleoyl-3-trimethylammonium-propane chloride (Avanti Polar Lipids, Inc. Alabaster, AL, US), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (Avanti Polar Lipids, Inc. Alabaster, AL, US) and 1,3-dioleoyloxy-2-(6-carboxyspermyl)propylamide (DOSPER).

Polyamines suitable as cationic trapping molecules are described in U.S. Pat. Nos. 6,379,965 and 6,372,499.

Payload Molecules

The particulate delivery system of the present invention is useful for *in vivo* or *in vitro* delivery of payload molecules including, but limited to, polynucleotides such as oligonucleotides, antisense constructs, siRNA, enzymatic RNA, and recombinant DNA constructs, including expression vectors.

In other preferred embodiments, the particulate delivery system of the present invention is useful for *in vivo* or *in vitro* delivery of payload molecules such as amino acids, peptides and proteins. By "protein" is meant a sequence of amino acids for which the chain length is sufficient to produce the higher levels of tertiary and/or quaternary structure. This is to distinguish from "peptides" or other small molecular weight drugs that do not have such structure. Typically, the protein herein will have a molecular weight of at least about 15-20 kD, preferably at least about 20 kD.

Examples of proteins encompassed within the definition herein include mammalian proteins, such as, e.g., growth hormone (GH), including human growth hormone, bovine growth hormone, and other members of the GH supergene family; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA); bombazine; thrombin; alpha tumor necrosis factor, beta tumor necrosis factor; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); serum albumin such as human serum albumin; mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; DNase; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; an integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-beta; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-beta1, TGF-beta2, TGF-beta3, TGF-beta4, or TGF-beta5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-D; insulin-like growth factor binding proteins;

CD proteins such as CD3, CD4, CD8, CD19 and CD20; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); T-cell receptors; surface membrane proteins; decay accelerating factor (DAF); a viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; immunoadhesins; antibodies; and biologically active fragments or variants of any of the above-listed polypeptides.

The members of the GH supergene family include growth hormone, prolactin, placental lactogen, erythropoietin, thrombopoietin, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-9, interleukin-10, interleukin-11, interleukin-12 (p35 subunit), interleukin-13, interleukin-15, oncostatin M, ciliary neurotrophic factor, leukemia inhibitory factor, alpha interferon, beta interferon, gamma interferon, omega interferon, tau interferon, granulocyte-colony stimulating factor, granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor, cardiotrophin-1 and other proteins identified and classified as members of the family.

The protein payload molecule is preferably essentially pure and desirably essentially homogeneous (i.e. free from contaminating proteins etc). "Essentially pure" protein means a composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight. "Essentially homogeneous" protein means a composition comprising at least about 99% by weight of protein, based on total weight of the composition. Proteins may be derived from naturally occurring sources or produced by recombinant technology. Proteins include protein variants produced by amino acid substitutions or by directed protein evolution (Kurtzman, A.L., et al., Advances in directed protein evolution by recursive genetic recombination: applications to therapeutic proteins, Curr Opin Biotechnol. 2001 12(4): 361-70) as well as derivatives, such as PEGylated proteins.

In certain embodiments, the protein is an antibody. The antibody may bind to any of the above-mentioned molecules, for example. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the HER receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mol, p150,95, VLA-4, ICAM-1, VCAM and alphav/beta3 integrin including either alpha or beta subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; protein C, etc.

In addition to peptides, polypeptides and polynucleotides, the particulate delivery system of the present invention is suitable for the delivery of smaller molecules, preferably for the delivery of pharmaceutically active agent, more preferably therapeutic small molecules. Suitable small molecule payloads for the delivery system of the present invention include contraceptive agents such as diethyl stilbestrol, 17-beta-estradiol, estrone, ethinyl estradiol, mestranol, and the like; progestins such as norethindrone, norgestryl, ethynodiol diacetate, lynestrenol, medroxyprogesterone acetate, dimethisterone, megestrol acetate, chlormadinone acetate, norgestimate, norethisterone, ethisterone, melengestrol, norethynodrel and the like; and spermicidal compounds such as nonylphenoxypolyoxyethylene glycol, benzethonium chloride, chlorindanol and the like. Preferably, for such steroidal payloads, a mixture of trapping molecules is used, comprising a sufficient amount of a detergent to solubilize the payload and a polymer to retain the payload within the yeast cell wall particle.

Other active agents that can be incorporated in the delivery system of the present invention include gastrointestinal therapeutic agents such as aluminum hydroxide, calcium carbonate, magnesium carbonate, sodium carbonate and the like; non-steroidal antifertility agents; parasympathomimetic agents; psychotherapeutic agents; major tranquilizers such as chloropromazine HCl, clozapine, mesoridazine, metiapine, reserpine, thioridazine and the like; minor tranquilizers such as chlordiazepoxide, diazepam, meprobamate, temazepam and the like; rhinological decongestants; sedative-hypnotics such as codeine, phenobarbital, sodium pentobarbital, sodium secobarbital and the like; other steroids such as testosterone and testosterone propionate; sulfonamides; sympathomimetic agents; vaccines; vitamins and nutrients such as the essential amino acids, essential fats and the like; antimalarials such as 4-aminoquinolines, 8-aminoquinolines, pyrimethamine and the like; anti-migraine agents such as mazindol, phentermine and the like; anti-Parkinson agents such as L-dopa; anti-spasmodics such as atropine, methscopolamine bromide and the like; antispasmodics and anticholinergic agents such as bile therapy, digestants, enzymes and the like; antitussives such as dextromethorphan, noscapine and the like; bronchodilators; cardiovascular agents such as anti-hypertensive compounds, *Rauwolfia* alkaloids, coronary vasodilators, nitroglycerin, organic nitrates, pentaerythritotetranitrate and the like; electrolyte replacements such as potassium chloride; ergotalkaloids such as ergotamine with and without caffeine, hydrogenated ergot alkaloids, dihydroergocristine methanesulfate, dihydroergocornine methanesulfonate, dihydroergokroyptine methanesulfate and combinations thereof; alkaloids such as atropine sulfate, Belladonna,

hyoscine hydrobromide and the like; analgesics; narcotics such as codeine, dihydrocodienone, meperidine, morphine and the like; non-narcotics such as salicylates, aspirin, acetaminophen, d-propoxyphene and the like.

In preferred embodiments, the system of the present invention is used to deliver antibiotics such as the cephalosporins, chloramphenical, gentamicin, kanamycin A, kanamycin B, the penicillins, ampicillin, streptomycin A, antimycin A, chloropamtheniol, metronidazole, oxytetracycline penicillin G, the tetracyclines, and the like. In preferred embodiments, the ability of the body's macrophages to inactivate pathogens is enhanced by the delivery of antibiotics, such as tetracycline, to the macrophages.

In other preferred embodiments, the present invention provides a system to deliver anti-cancer agents; anti-convulsants such as mephenytoin, phenobarbital, trimethadione; anti-emetics such as thiethylperazine; antihistamines such as chlorophinazine, dimenhydrinate, diphenhydramine, perphenazine, tripelennamine and the like; anti-inflammatory agents such as hormonal agents, hydrocortisone, prednisolone, prednisone, non-hormonal agents, allopurinol, aspirin, indomethacin, phenylbutazone and the like; prostaglandins; cytotoxic drugs such as thiotepa, chlorambucil, cyclophosphamide, melphalan, nitrogen mustard, methotrexate and the like.

Vaccines

In preferred embodiments, the particulate delivery system of the present invention is useful in providing oral delivery of vaccines. In preferred embodiments, the system is used to deliver antigens, such as antigens of such microorganisms as *Neisseria gonorrhea*, *Mycobacterium tuberculosis*, *Herpes virus* (*humonis*, types 1 and 2), *Candida albicans*, *Candida tropicalis*, *Trichomonas vaginalis*, *Haemophilus vaginalis*, Group B *Streptococcus sp.*, *Microplasma hominis, Hemophilus ducreyi, Granuloma inguinale, Lymphopathia venereum, Treponema pallidum, Brucella abortus. Brucella melitensis, Brucella suis, Brucella canis, Campylobacter fetus, Campylobacter fetus intestinalis, Leptospira pomona, Listeria monocytogenes, Brucella ovis,* equine herpes virus 1, equine arteritis virus, IBR-IBP virus, BVD-MB virus, *Chlamydia psittaci, Trichomonas foetus, Toxoplasma gondii, Escherichia coli, Actinobacillus equuli, Salmonella abortus ovis, Salmonella abortus equi, Pseudomonas aeruginosa, Corynebacterium equi, Corynebacterium pyogenes, Actinobaccilus seminis, Mycoplasma bovigenitalium, Aspergillus fumigatus, Absidia ramosa, Trypanosoma equiperdum,*

Babesia caballi, Clostridium tetani, Clostridium botulinum and the like. In other embodiments, the system can be used to deliver neutralizing antibodies that counteract the above microorganisms.

In other embodiments, the system can be used to deliver enzymes such as ribonuclease, neuramidinase, trypsin, glycogen phosphorylase, sperm lactic dehydrogenase, sperm hyaluronidase, adenossinetriphosphatase, alkaline phosphatase, alkaline phosphatase esterase, amino peptidase, trypsin chymotrypsin, amylase, muramidase, acrosomal proteinase, diesterase, glutamic acid dehydrogenase, succinic acid dehydrogenase, beta-glycophosphatase, lipase, ATP-ase alpha-peptate gamma-glutamylotranspeptidase, sterol-3-beta-ol-dehydrogenase, DPN-di-aprorase.

In preferred embodiments, the system can deliver antigens of bioterrorism critical biological agents, including Category A agents such as variola major (smallpox), *Bacillus anthracis* (anthrax), *Yersinia pestis* (plague), *Clostridium botulinum* toxin (botulism), *Francisella tularensis* (tularaemia), filoviruses (Ebola hemorrhagic fever, Marburg hemorrhagic fever), arenaviruses (Lassa (Lassa fever),Junin (Argentine hemorrhagic fever) and related viruses); Category B agents such as *Coxiella burnetti* (Q fever), *Brucella* species (brucellosis), *Burkholderia mallei* (glanders), alphaviruses (Venezuelan encephalomyelitis, eastern & western equine encephalomyelitis), ricin toxin from *Ricinus communis* (castor beans), epsilon toxin of *Clostridium perfringens*; *Staphylococcus* enterotoxin B, *Salmonella* species, *Shigella dysenteriae*, *Escherichia coli* strain O157:H7, *Vibrio cholerae*, *Cryptosporidium parvum*; and Category C agents such as nipah virus, hantaviruses, tickborne hemorrhagic fever viruses, tickborne encephalitis viruses, yellow fever, and multidrug-resistant tuberculosis.

In preferred embodiments, the system can be used to deliver inactivated antigenic toxins, such as anatoxin antigens, including toxoids (inactivated but antigenic toxins), and toxoid conjugates. In preferred embodiments, the toxoid is an inactivated microbial toxin. In other embodiments, the toxoid is an inactivated plant toxin. In further embodiments, the toxoid is an inactivated animal toxin. In certain embodiments, the system can be used to deliver toxoids such as pertussis toxoid, *Corynebacterium diphtheriae* toxoid, tetanus toxoid, *Haemophilus influenzae* type b-tetanus toxoid conjugate, *Clostridium botulinum* D toxoid, *Clostridium botulinum* E toxoid, toxoid produced from Toxin A of *Clostridium difficile*, *Vibrio cholerae* toxoid, *Clostridium perfringens* Types C and D toxoid, *Clostridium chauvoei* toxoid, *Clostridium novyi* (Type B) toxoid, *Clostridium septicum* toxoid, recombinant HIV tat IIIB

toxoid, *Staphylococcus* toxoid, *Actinobacillus pleuropneumoniae* Apx I toxoid, , *Actinobacillus pleuropneumoniae* Apx II toxoid, , *Actinobacillus pleuropneumoniae* Apx III toxoid, *Actinobacillus pleuropneumoniae* outer membrane protein (OMP) toxoid, *Pseudomonas aeruginosa* elastase toxoid, snake venom toxoid, ricin toxoid, *Mannheimia haemolytica* toxoid, *Pasteurella multocida* toxoid, *Salmonella typhimurium* toxoid, *Pasteurella multocida* toxoid, and *Bordetella bronchiseptica* toxoid.

Techniques of making a toxoid from a corresponding toxin, e.g. chemical treatment with formaldehyde or aluminum salts or gamma irradiation, are known in the art. Recombinant methods of converting a toxin to a toxoid are also known (Fromen-Romano, C., et al., Transformation of a non-enzymatic toxin into a toxoid by genetic engineering, Protein Engineering vol.10 no.10 pp.1213–1220, 1997). In preferred embodiments, the system of the present invention can be used to deliver a recombinant toxoid. In other preferred embodiments, the system of the present invention can be used to deliver an expression vector encoding a recombinant toxoid.

In order to produce a genetic vaccine to protect against pathogen infection, genetic material which encodes immunogenic proteins against which a protective immune response can be mounted, must be included in the nucleic acid composition. Whether the pathogen infects intracellularly, for which the present invention is particularly useful, or extracellularly, it is unlikely that all pathogen antigens will elicit a protective response. Because DNA and RNA are both relatively small and can be produced relatively easily, the present invention provides the additional advantage of allowing for vaccination with multiple pathogen antigens. The nucleic acid composition used in the genetic vaccine can include genetic material that encodes many pathogen antigens. For example, several viral genes may be included in a single construct, thereby providing multiple targets. In addition, multiple inoculants which can be delivered to different cells in an individual can be prepared to collectively include, in some cases, a complete or, more preferably, an incomplete, e.g., nearly complete set of genes in the vaccine. For example, a complete set of viral genes may be administered using two constructs which each contain a different half of the genome which are administered at different sites. Thus, an immune response may be invoked against each antigen without the risk of an infectious virus being assembled. This allows for the introduction of more than a single antigen target and can eliminate the requirement that protective antigens be identified.

In accordance with the present invention there is also provided a method of conferring a broad based protective immune response against hyperproliferating cells that are characteristic of hyperproliferative diseases, as well as a method of treating individuals suffering from hyperproliferative diseases. As used herein, the term "hyperproliferative diseases" is meant to refer to those diseases and disorders characterized by hyperproliferation of cells. Examples of hyperproliferative diseases include all forms of cancer and psoriasis.

It has been discovered that introduction of a nucleic acid composition that includes a nucleotide sequence which encodes an immunogenic "hyperproliferating cell"-associated protein into the cells of an individual, results in the production of those proteins in the vaccinated cells of an individual. As used herein, the term "hyperproliferative-associated protein" is meant to refer to proteins that are associated with a hyperproliferative disease. To immunize against hyperproliferative diseases, a nucleic acid composition that includes a nucleotide sequence which encodes a protein that is associated with a hyperproliferative disease is administered to an individual.

In order for the hyperproliferative-associated protein to be an effective immunogenic target, it must be a protein that is produced exclusively or at higher levels in hyperproliferative cells as compared to normal cells. Target antigens include such proteins, fragments thereof and peptides which comprise at least an epitope found on such proteins. In some cases, a hyperproliferative-associated protein is the product of a mutation of a gene that encodes a protein. The mutated gene encodes a protein which is nearly identical to the normal protein except it has a slightly different amino acid sequence which results in a different epitope not found on the normal protein. Such target proteins include those which are proteins encoded by oncogenes such as myb, myc, fyn, and the translocation genes bcr/abl, ras, src, P53, neu, trk and EGRF. In addition to oncogene products as target antigens, target proteins for anti-cancer treatments and protective regimens include variable regions of antibodies made by B cell lymphomas, and variable regions of T cell receptors of T cell lymphomas which, in some embodiments, are also used as target antigens for autoimmune diseases. Other tumor-associated proteins can be used as target proteins, such as proteins which are found at higher levels in tumor cells, including the protein recognized by monoclonal antibody 17-1A and folate binding proteins.

While the present invention may be used to immunize an individual against one or more of several forms of cancer, the present invention is particularly useful to prophylactically

immunize an individual who is predisposed to develop a particular cancer or who has had cancer and is therefore susceptible to a relapse. Developments in genetics and biotechnology, as well as epidemiology, allow for the determination of probability and risk assessment for the development of cancer in an individual. Using genetic screening and/or family health histories, it is possible to predict the probability that a particular individual has for developing any one of several types of cancer.

Similarly, those individuals who have already developed cancer and who have been treated to remove the cancer, or are otherwise in remission, are particularly susceptible to relapse and reoccurrence. As part of a treatment regimen, such individuals can be immunized against the cancer that they have been diagnosed as having had in order to combat such a recurrence. Thus, once it is known that individuals have had a type of cancer and are at risk of a relapse, they can be immunized in order to prepare their immune systems to combat any future appearance of the cancer.

The present invention also provides a method of treating individuals suffering from hyperproliferative diseases. In such methods, the introduction of peptide, protein, carbohydrate or nucleic acid compositions and combinations thereof serves as an immunotherapeutic, directing and promoting the immune system of the individual to combat hyperproliferative cells that produce the target protein.

The present invention provides a method of treating individuals suffering from autoimmune diseases and disorders by conferring a broad based protective immune response against targets that are associated with autoimmunity, including cell receptors and cells which produce "self"-directed antibodies.

T cell mediated autoimmune diseases include Rheumatoid arthritis (RA), multiple sclerosis (MS), Sjogren's syndrome, sarcoidosis, insulin dependent diabetes mellitus (IDDM), autoimmune thyroiditis, reactive arthritis, ankylosing spondylitis, scleroderma, polymyositis, dermatomyositis, psoriasis, vasculitis, Wegener's granulomatosis, Crohn's disease and ulcerative colitis. Each of these diseases is characterized by T cell receptors that bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of the T cells would elicit an immune response including CTLs to eliminate those T cells.

In RA, several specific variable regions of T cell receptors (TCRs) which are involved in the disease have been characterized. These TCRs include Vβ-3, Vβ-14, Vβ-17 and Vα-17. Thus,

vaccination with a composition composed of peptide, protein, carbohydrate or nucleic acid compositions and combinations thereof that delivers or encodes at least one of these proteins will elicit an immune response that will target T cells involved in RA. See: Howell, M. D., et al., 1991 Proc. Natl. Acad. Sci. USA 88:10921-10925; Paliard, X., et al., 1991 Science 253:325-329; Williams, W. V., et al.., 1992 J. Clin. Invest. 90:326-333; each of which is incorporated herein by reference.

In MS, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include Vβ-7 and Vα-10. Thus, vaccination with a composition composed of peptide, protein, carbohydrate or nucleic acid compositions and combinations thereof that delivers or encodes at least one of these proteins will elicit an immune response that will target T cells involved in MS. See: Wucherpfennig, K. W., et al., 1990 Science 248:1016-1019; Oksenberg, J. R., et al., 1990 Nature 345:344-346; each of which is incorporated herein by reference.

In scleroderma, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include Vβ-6, Vβ-8, Vβ-14 and Vα-16, Vα-3C, Vα-7, Vα-14, Vα-15, Vα-16, Vα-28 and Vα-12. Thus, vaccination with a composition composed of peptide, protein, carbohydrate or nucleic acid compositions and combinations thereof that delivers or encodes for at least one of these proteins will elicit an immune response that will target T cells involved in scleroderma.

In order to treat patients suffering from a T cell mediated autoimmune disease, particularly those for which the variable region of the TCR has yet to be characterized, a synovial biopsy can be performed. Samples of the T cells present can be taken and the variable region of those TCRs identified using standard techniques. Vaccines can be prepared using this information.

B cell mediated autoimmune diseases include Lupus (SLE), Grave's disease, myasthenia gravis, autoimmune hemolytic anemia, autoimmune thrombocytopenia, asthma, cryoglobulinemia, primary biliary sclerosis and pernicious anemia. Each of these diseases is characterized by antibodies which bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of such antibodies would elicit an immune response including CTLs to eliminate those B cells that produce the antibody.

In order to treat patients suffering from a B cell mediated autoimmune disease, the variable region of the antibodies involved in the autoimmune activity must be identified. A biopsy can be performed and samples of the antibodies present at a site of inflammation can be taken. The variable region of those antibodies can be identified using standard techniques. Vaccines can be prepared using this information.

In the case of SLE, one antigen is believed to be DNA. Thus, in patients to be immunized against SLE, their sera can be screened for anti-DNA antibodies and a vaccine can be prepared which includes nucleic acid compositions that encode the variable region of such anti-DNA antibodies found in the sera.

Common structural features among the variable regions of both TCRs and antibodies are well known. The DNA sequence encoding a particular TCR or antibody can generally be found following well known methods such as those described in Kabat, et al. 1987 Sequence of Proteins of Immunological Interest U.S. Department of Health and Human Services, Bethesda Md., which is incorporated herein by reference. In addition, a general method for cloning functional variable regions from antibodies can be found in Chaudhary, V. K., et al., 1990 Proc. Natl. Acad. Sci. USA 87:1066, which is incorporated herein by reference.

Gene Therapy

In preferred embodiments, the present invention provides compositions and methods for the treatment of genetic disorders or conditions having a genetic component. In further preferred embodiments, the present invention provides compositions useful for the manufacture of pharmaceutical products for the treatment of genetic disorders or conditions having a genetic component.

The Human Genome Project has increased our knowledge of the genetic basis of disease. See, generally, http://www.ornl.gov/sci/techresources/Human_Genome/medicine/assist.shtml.

Both environmental and genetic factors have roles in the development of any disease. A genetic disorder is a disease caused by abnormalities in an individual's genetic material (genome). There are four different types of genetic disorders: (1) single-gene, (2) multifactorial, (3) chromosomal, and (4) mitochondrial.

(1) Single-gene (also called Mendelian or monogenic) - This type is caused by changes or mutations that occur in the DNA sequence of one gene. Genes code for proteins, the molecules that carry out most of the work, perform most life functions, and even make up the majority of

cellular structures. When a gene is mutated so that its protein product can no longer carry out its normal function, a disorder can result. There are more than 6,000 known single-gene disorders, which occur in about 1 out of every 200 births. Some examples are cystic fibrosis, sickle cell anemia, Marfan syndrome, Huntington's disease, and hereditary hemochromatosis.

(2) Multifactorial (also called complex or polygenic) - This type is caused by a combination of environmental factors and mutations in multiple genes. For example, different genes that influence breast cancer susceptibility have been found on chromosomes 6, 11, 13, 14, 15, 17, and 22. Its more complicated nature makes it much more difficult to analyze than single-gene or chromosomal disorders. Some of the most common chronic disorders are multifactorial disorders. Examples include heart disease, high blood pressure, Alzheimer's disease, arthritis, diabetes, cancer, and obesity. Multifactorial inheritance also is associated with heritable traits such as fingerprint patterns, height, eye color, and skin color.

(3) Chromosomal - Chromosomes, distinct structures made up of DNA and protein, are located in the nucleus of each cell. Because chromosomes are carriers of genetic material, such abnormalities in chromosome structure as missing or extra copies or gross breaks and rejoinings (translocations), can result in disease. Some types of major chromosomal abnormalities can be detected by microscopic examination. Down syndrome or trisomy 21 is a common disorder that occurs when a person has three copies of chromosome 21.

(4) Mitochondrial - This relatively rare type of genetic disorder is caused by mutations in the nonchromosomal DNA of mitochondria. Mitochondria are small round or rod-like organelles that are involved in cellular respiration and found in the cytoplasm of plant and animal cells. Each mitochondrion may contain 5 to 10 circular pieces of DNA.

In preferred embodiments, the particulate delivery system of the present invention is used to administer at least one polynucleotide comprising a compensating gene. In other preferred embodiments, the particulate delivery system of the present invention is used to administer at least one polynucleotide encoding a gene product of a missing gene, wherein the expression of the gene product is useful in the treatment of the genetic disorder or the genetic component of a condition. In preferred embodiments, the particulate delivery system of the present invention including the desired payload molecule is useful for the manufacture of a pharmaceutical product for the treatment of genetic disorder or the genetic component of a condition. Such pharmaceutical products are suitably administered orally, rectally, parenterally, (for example, intravenously, intramuscularly, or subcutaneously) intracisternally, intravaginally,

intraperitoneally, intravesically, locally (for example, powders, ointments or drops), or as a buccal or nasal spray. The pharmaceutical products are preferably administered orally, buccally, and parenterally, more preferably orally. Particles loaded with different payloads, e.g. a polynucleotide, a polynucleotide expression vector or a small molecule therapeutic can be mixed in the appropriate proportions and administered together, e.g., in a capsule, for combination therapy.

In aspects of the present invention that relate to gene therapy, the nucleic acid compositions contain either compensating genes or genes that encode therapeutic proteins. Examples of compensating genes include a gene that encodes dystrophin or a functional fragment, a gene to compensate for the defective gene in patients suffering from cystic fibrosis, a gene to compensate for the defective gene in patients suffering from ADA, and a gene encoding Factor VIII. Examples of genes encoding therapeutic proteins include genes which encodes erythropoietin, interferon, LDL receptor, GM-CSF, IL-2, IL-4 and TNF. Additionally, nucleic acid compositions which encode single chain antibody components which specifically bind to toxic substances can be administered. In some preferred embodiments, the dystrophin gene is provided as part of a mini-gene and used to treat individuals suffering from muscular dystrophy. In some preferred embodiments, a mini-gene which contains coding sequence for a partial dystrophin protein is provided. Dystrophin abnormalities are responsible for both the milder Becker's Muscular Dystrophy (BMD) and the severe Duchenne's Muscular Dystrophy (DMD). In BMD dystrophin is made, but it is abnormal in either size and/or amount. The patient is mild to moderately weak. In DMD no protein is made and the patient is wheelchair-bound by age 13 and usually dies by age 20. In some patients, particularly those suffering from BMD, partial dystrophin protein produced by expression of a mini-gene delivered according to the present invention can provide improved muscle function.

In preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of genetic disorders and conditions believed to have a genetic component, such as Aarskog-Scott syndrome, Aase syndrome, achondroplasia, acrodysostosis, addiction, adreno-leukodystrophy, albinism, ablepharon-macrostomia syndrome, alagille syndrome, alkaptonuria, alpha-1 antitrypsin deficiency, Alport's syndrome, Alzheimer's disease, asthma, autoimmune polyglandular syndrome, androgen insensitivity syndrome, Angelman syndrome, ataxia, ataxia telangiectasia, atherosclerosis, attention deficit hyperactivity disorder (ADHD), autism, baldness, Batten disease, Beckwith-

Wiedemann syndrome, Best disease, bipolar disorder, brachydactyly, breast cancer, Burkitt lymphoma, chronic myeloid leukemia, Charcot-Marie-Tooth disease, Crohn's disease, cleft lip, Cockayne syndrome, Coffin Lowry Syndrome, colon cancer, congenital adrenal hyperplasia (CAH), Cornelia de Lange Syndrome, Costello Syndrome, Cowden Syndrome, Craniofrontonasal Dysplasia, Crigler-Najjar Syndrome, Creutzfeldt-Jakob Disease (CJD), cystic fibrosis, deafness, depression, diabetes, diastrophic dysplasia, DiGeorge Syndrome, Down's Syndrome, dyslexia, Duchenne muscular dystrophy, Dubowitz Syndrome, ectodermal dysplasia, Ellis-van Creveld syndrome, Ehlers-Danlos, Epidermolysis Bullosa (EB), epilepsy, essential tremor, familial hypercholesterolemia, familial Mediterranean fever, Fragile X Syndrome, Friedreich's ataxia, Gaucher disease, glaucoma, glucose galactose malabsorption, glutaricaciduria, gyrate atrophy, Goldberg Shprintzen Syndrome (velocardiofacial syndrome), Gorlin Syndrome, Hailey-Hailey Disease, hemihypertrophy, hemochromatosis, hemophilia, hereditary motor and sensory neuropathy (HMSN), hereditary non polyposis colorectal cancer (HNPCC), Huntington's disease, immunodeficiency with hyper-IgM, juvenile onset diabetes, Klinefelter's Syndrome, Kabuki Syndrome, Leigh's Disease (or Syndrome), Long QT Syndrome, lung cancer, malignant melanoma, manic depression, Marfan Syndrome, Menkes syndrome, miscarriage, mucopolysaccharide disease, multiple endocrine neoplasia, multiple sclerosis, muscular dystrophy, myotrophic lateral sclerosis, myotonic dystrophy, neurofibromatosis, Niemann-Pick disease, Noonan Syndrome, obesity, ovarian cancer, p53 tumor suppressor, pancreatic cancer, Parkinson disease, paroxysmal nocturnal hemoglobinuria, Pendred syndrome, peroneal muscular atrophy, phenylketonuria (PKU), polycystic kidney disease, Prader-Willi Syndrome, primary biliary cirrhosis, prostate cancer, REAR Syndrome, Refsum disease, retinitis pigmentosa, retinoblastoma, Rett Syndrome, Sanfilippo Syndrome, schizophrenia, severe combined immunodeficiency, sickle cell anemia, spina bifida, spinal muscular atrophy, spinocerebellar atrophy, SRY: sex determination, Sudden Adult Death Syndrome, Tangier disease, Tay-Sachs disease, thrombocytopenia absent radius syndrome, Townes-Brocks Syndrome, tuberous sclerosis, Turner syndrome, Usher syndrome, von Hippel-Lindau syndrome, Waardenburg syndrome, Weaver syndrome, Werner syndrome, Williams syndrome, Wilson's Disease, xeroderma pigmentosum and Zellweger syndrome.

In other preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of genetic disorders and conditions believed to have a genetic component that are manifested as metabolic disorders, such as protein-

related disorders, including Sickle-Cell Anemia and beta-Thalassemias, alpha-Thalassemias, Marfan's Syndrome, Ehlers-Danlos Type I, Ehlers-Danlos Type II, Ehlers-Danlos Type III, Ehlers-Danlos Type IV autosomal dominant, Ehlers-Danlos Type IV autosomal recessive, Ehlers-Danlos Type IV-D, Ehlers-Danlos Type V, Ehlers-Danlos Type VI, Ehlers-Danlos Type VII autosomal dominant, Ehlers-Danlos Type VII autosomal recessive, Ehlers-Danlos Type VIII. Ehlers-Danlos with Platelet Dysfunction, Cutis Laxa, Cutis Laxa recessive Type I, Occipital Horn Syndrome Cutis Laxa, X-linked, Osteogenesis Imperfecta Type I, Osteogenesis Imperfecta Type I-C, Osteogenesis Imperfecta Silent Type II/III, Osteogenesis Imperfecta Type IV, Osteogenesis Imperfecta Neonatal Lethal form, and Osteogenesis Imperfecta progressively deforming.

In further preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of genetic disorders of the clotting system, such as afibrinogenemia , complete loss of fibrinogen, Factor I; dysfibrinogenemia dysfunctional fibrinogen, Factor I; Factor II disorders; tissue factor deficiency; Factor V deficiency, labile Factor deficiency, Factor VII deficiency, Factor VIII deficiency (Hemophilia A), Factor IX deficiency (Hemophilia B), Factor X deficiency, Factor XI deficiency, Rosenthal Syndrome, Plasma Thromboplastin Antecedent (PTA) deficiency, Factor XII deficiency, Hageman factor deficiency, Factor XIII deficiency, Factor V & VIII Combined deficiency, Factor VIII & IX combined deficiency, Factor IX & XI Combined deficiency, Protein C deficiency, Protein S deficiency, thrombophilia, antithrombin III deficiency, giant platelet syndrome, platelet glycoprotein Ib deficiency, von Willebrand disease, Fletcher Factor deficiency and prekallikrein deficiency.

In further preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of glycogen storage disorders, such as Type 0, Type I (von Gierke's disease), Type Ib, Type Ic, Type II (Pompe disease), Type IIb (Danon disease), Type III (Cori disease or Forbes disease), Type IV (Andersen disease), Type V (McArdle disease), Type VI (Hers disease), Type VII (Tarui disease), Type VIII, Type IX, and Type XI (Fanconi-Bickel syndrome).

In yet further preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of defects in fructose, galactose and glycerol metabolism, such as hereditary fructose intolerance, aldolase B deficiency;

fructosuria, hepatic fructokinase deficiency; classic galactosemia, galactose epimerase deficiency; galactokinase deficiency; hyperglycerolemia and glycerol kinase deficiency.

In yet further preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of defects in cholesterol and lipoprotein metabolism, such as apolipoprotein(a) - Lp(a), hyperlipoproteinemia Type I; hyperlipoproteinemia Type Ib; apolipoprotein C-II deficiency; hyperlipoproteinemia Type Ic, chylomicronemia; familial hypercholesterolemia, Type II hyperlipoproteinemia; hyperlipoproteinemia Type II, familial hyperbetalipoproteinemia; hyperlipoproteinemia Type III, apolipoprotein E deficiency; hyperlipoproteinemia Type IV; hyperlipoproteinemia Type V; familial LCAT deficiency; Wolman disease; lipoprotein lipase deficiency; familial hypertriglyceridemia; hyperlipidemia Type V; hyperlipidemia Type VI; familial ligand-defective apo-B; familial hyperalphalipoproteinemia; hypobetalipoproteinemia, apolipoprotein B-100 deficiency; abetalipoproteinemia, Kornzweig syndrome; and Tangier Disease, familial high-density lipoprotein deficiency.

In yet further preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of mucopolysaccharide and glycolipid disorders, such as Type I H mucopolysaccharidosis (Hurler syndrome), Type I S mucopolysaccharidosis (Scheie syndrome), Type I H/S mucopolysaccharidosis (Hurler/ Scheie syndrome), Type II mucopolysaccharidosis (Hunter's syndrome), Type III mucopolysaccharidoses (Sanfilippo Type A, Sanfilippo Type B, Sanfilippo Type C, Sanfilippo Type D), Type IV mucopolysaccharidosis (Morquio's Type A, Morquio's Type B), Type VI mucopolysaccharidosis (Maroteaux-Lamy Syndrome) and Type VII mucopolysaccharidosis (Sly Syndrome).

In other preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of disorders of glycosphingolipid metabolism, such as GM1 gangliosidoses, including generalized GM1 Type II, juvenile form; generalized GM1 Type III, adult form; GM2 gangliosidosis, Sandhoff-Jatzkewitz disease; GM3 gangliosidoses, Tay-Sachs disease, Tay-Sachs AB variant, Gaucher disease, Niemann-Pick Disease, Types A, B, C1, C2 and D, Schindler disease, Fabry disease, lactosylceramidosis, Farber disease, Krabbe disease, multiple sulfatase deficiency, Austin disease, metachromic leukodystrophy, and sulfatide lipodosis.

In other preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of oligosaccharidoses such as fucosidosis, mucolipodosis VI, sialolipidosis, alpha-mannosidosis, beta-mannosidosis, sialidoses Types I and II, galactosialidosis, Goldberg syndrome and aspartylglucosaminuria.

In other preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of disorders of lysosomal enzyme transport such as mucolipidosis I, sialidosis; mucolipodosis II, I-cell disease; and mucolipodosis III, pseudo-Hurler polydystrophy.

In other preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of defects in amino acid and organic acid metabolism such as phenylketonuria; Type I tyrosinemia, tyrosinosis; Type II tyrosinemia, Richner-Hanhart syndrome; Type III tyrosinemia; alcaptonuria; homocystinuria; histidinemia; maple syrup urine disease (MSUD); MSUD Type Ib, MSUD type II; methylmalonic aciduria; non-ketonic hyperglycinemia Type I (NKHI) and hyperlysinemia.

In other preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of urea cycle defects such as hyperammonemias; carbamoyl phosphate synthetase I (CPS-I) deficiency; ornithine transcarbamylase (OTC) deficiency; N-acetylglutamate synthetase deficiency; argininosuccinic aciduria, argininosuccinate lyase deficiency; hyperargininemia, arginase deficiency; citrullinemia, argininosuccinate synthetase deficiency and ornithine aminotransferase deficiency. In other preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of defects in amino acid transport such as cystinuria Type I; cystinuria Type III; Hartnup disease and hyperammonemia-hyperornithinemia-homocitrullinuria (HHH) syndrome. In other preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of porphyrias and bilirubinemias such as congenital erythropoietic porphyria (CEP); erythropoietic protoporphyria (EPP); ALA dehydratase deficiency porphyria (ADP); acute intermittent porphyria (AIP); hereditary coproporphyria (HCP); variegate porphyria (VP); porphyria cutanea tarda (PCT); hepatoerythropoietic porphyria (HEP); Gilbert Syndrome; Crigler-Najjar Syndrome, Types I and I; Dubin-Johnson syndrome and Rotor syndrome.

In other preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of errors in fatty acid metabolism such as

very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD); long-chain acyl-CoA dehydrogenase deficiency (LCAD); medium-chain acyl-CoA dehydrogenase deficiency (MCAD); short-chain acyl-CoA dehydrogenase deficiency (SCAD; carnitine translocase deficiency; carnitine palmitoyltransferase I (CPT I) deficiency and carnitine palmitoylransferase II (CPT II) deficiency. In other preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of defects in nucleotide metabolism such as Lesch-Nyhan syndrome; Severe Combined Immunodeficiency Disease (SCID), due to adenosine deaminase (ADA) deficiency; gout; renal lithiasis, due to adenine phosphoribosyltransferase (APRT) deficiency; xanthinuria, due to xanthine oxidase deficiency; orotic aciduria,Types I & I and ornithine transcarbamoylase deficiency.

In other preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of disorders in metal metabolism and transport such as Wilson disease, Menkes disease, occipital horn syndrome and hemochromatosis. In other preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of disorders in peroxisomes such as Zellweger syndrome, X-linked adreoleukodystrophy, neonatal adrenoleukodystophy (NALD), rhizomelic chondrodysplasia punctata (RCDP) and infantile Refsum's disease (IRD). In other preferred embodiments, the particulate delivery system of the present invention provides compositions and methods for the treatment of disorders associated with defective DNA repair such as ataxia telangiectasia (AT), xeroderma pigmentosum (XP), Cockayne syndrome, Bloom syndrome and Fanconi anemia.

Routes of Administration

Routes of administration include but are not limited to oral; buccal, sublingual, pulmonary, transdermal, transmucosal, as well as subcutaneous, intraperitoneal, intravenous, and intramuscular injection. Preferred routes of administration are oral; buccal, sublingual, pulmonary and transmucosal.

The particulate delivery system of the present invention is administered to a patient in a therapeutically effective amount. The particulate delivery system can be administered alone or as part of a pharmaceutically acceptable composition. In addition, a compound or composition can be administered all at once, as for example, by a bolus injection, multiple times, such as by a series of tablets, or delivered substantially uniformly over a period of time, as for example, using

a controlled release formulation. It is also noted that the dose of the compound can be varied over time. The particulate delivery system can be administered using an immediate release formulation, a controlled release formulation, or combinations thereof. The term "controlled release" includes sustained release, delayed release, and combinations thereof.

A pharmaceutical composition of the invention can be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient that would be administered to a patient or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

The relative amounts of the active ingredient, the pharmaceutically acceptable carrier, and any additional ingredients in a pharmaceutical composition of the invention will vary, depending upon the identity, size, and condition of the human treated and further depending upon the route by which the composition is to be administered. By way of example, the composition can comprise between 0.1% and 100% (w/w) active ingredient. A unit dose of a pharmaceutical composition of the invention will generally comprise from about 100 milligrams to about 2 grams of the active ingredient, and preferably comprises from about 200 milligrams to about 1.0 gram of the active ingredient.

In addition, a particulate delivery system of the present invention can be administered alone, in combination with a particulate delivery system with a different payload, or with other pharmaceutically active compounds. The other pharmaceutically active compounds can be selected to treat the same condition as the particulate delivery system or a different condition.

If the patient is to receive or is receiving multiple pharmaceutically active compounds, the compounds can be administered simultaneously or sequentially in any order. For example, in the case of tablets, the active compounds may be found in one tablet or in separate tablets, which can be administered at once or sequentially in any order. In addition, it should be recognized that the compositions can be different forms. For example, one or more compounds may be delivered via a tablet, while another is administered via injection or orally as a syrup.

Another aspect of the invention relates to a kit comprising a pharmaceutical composition of the invention and instructional material. Instructional material includes a publication, a recording, a diagram, or any other medium of expression which is used to communicate the usefulness of the pharmaceutical composition of the invention for one of the purposes set forth

herein in a human. The instructional material can also, for example, describe an appropriate dose of the pharmaceutical composition of the invention. The instructional material of the kit of the invention can, for example, be affixed to a container which contains a pharmaceutical composition of the invention or be shipped together with a container which contains the pharmaceutical composition. Alternatively, the instructional material can be shipped separately from the container with the intention that the instructional material and the pharmaceutical composition be used cooperatively by the recipient.

The invention also includes a kit comprising a pharmaceutical composition of the invention and a delivery device for delivering the composition to a human. By way of example, the delivery device can be a squeezable spray bottle, a metered-dose spray bottle, an aerosol spray device, an atomizer, a dry powder delivery device, a self-propelling solvent/powder-dispensing device, a syringe, a needle, a tampon, or a dosage- measuring container. The kit can further comprise an instructional material as described herein.

For example, a kit may comprise two separate pharmaceutical compositions comprising respectively a first composition comprising a particulate delivery system and a pharmaceutically acceptable carrier; and composition comprising second pharmaceutically active compound and a pharmaceutically acceptable carrier. The kit also comprises a container for the separate compositions, such as a divided bottle or a divided foil packet. Additional examples of containers include syringes, boxes, bags, and the like. Typically, a kit comprises directions for the administration of the separate components. The kit form is particularly advantageous when the separate components are preferably administered in different dosage forms (e.g., oral and parenteral), are administered at different dosage intervals, or when titration of the individual components of the combination is desired by the prescribing physician.

An example of a kit is a blister pack. Blister packs are well known in the packaging industry and are being widely used for the packaging of pharmaceutical unit dosage forms (tablets, capsules, and the like). Blister packs generally consist of a sheet of relatively stiff material covered with a foil of a preferably transparent plastic material. During the packaging process recesses are formed in the plastic foil. The recesses have the size and shape of the tablets or capsules to be packed. Next, the tablets or capsules are placed in the recesses and a sheet of relatively stiff material is sealed against the plastic foil at the face of the foil which is opposite from the direction in which the recesses were formed. As a result, the tablets or capsules are sealed in the recesses between the plastic foil and the sheet. Preferably the strength of the sheet is

such that the tablets or capsules can be removed from the blister pack by manually applying pressure on the recesses whereby an opening is formed in the sheet at the place of the recess. The tablet or capsule can then be removed via said opening.

It may be desirable to provide a memory aid on the kit, e.g., in the form of numbers next to the tablets or capsules whereby the numbers correspond with the days of the regimen that the tablets or capsules so specified should be ingested. Another example of such a memory aid is a calendar printed on the card, e.g., as follows "First Week, Monday, Tuesday, . . . etc. . . . Second Week, Monday, Tuesday," etc. Other variations of memory aids will be readily apparent. A "daily dose" can be a single tablet or capsule or several pills or capsules to be taken on a given day. Also, a daily dose of a particulate delivery system composition can consist of one tablet or capsule, while a daily dose of the second compound can consist of several tablets or capsules and vice versa. The memory aid should reflect this and assist in correct administration.

In another embodiment of the present invention, a dispenser designed to dispense the daily doses one at a time in the order of their intended use is provided. Preferably, the dispenser is equipped with a memory aid, so as to further facilitate compliance with the dosage regimen. An example of such a memory aid is a mechanical counter, which indicates the number of daily doses that have been dispensed. Another example of such a memory aid is a battery-powered micro-chip memory coupled with a liquid crystal readout, or audible reminder signal which, for example, reads out the date that the last daily dose has been taken and/or reminds one when the next dose is to be taken.

A particulate delivery system composition, optionally comprising other pharmaceutically active compounds, can be administered to a patient either orally, rectally, parenterally, (for example, intravenously, intramuscularly, or subcutaneously) intracisternally, intravaginally, intraperitoneally, intravesically, locally (for example, powders, ointments or drops), or as a buccal or nasal spray.

Parenteral administration of a pharmaceutical composition includes any route of administration characterized by physical breaching of a tissue of a human and administration of the pharmaceutical composition through the breach in the tissue. Parenteral administration thus includes administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral

administration includes subcutaneous, intraperitoneal, intravenous, intraarterial, intramuscular, or intrasternal injection and intravenous, intraarterial, or kidney dialytic infusion techniques.

Compositions suitable for parenteral injection comprise the active ingredient combined with a pharmaceutically acceptable carrier such as physiologically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions, or emulsions, or may comprise sterile powders for reconstitution into sterile injectable solutions or dispersions. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents, or vehicles include water, isotonic saline, ethanol, polyols (propylene glycol, polyethylene glycol, glycerol, and the like), suitable mixtures thereof, triglycerides, including vegetable oils such as olive oil, or injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersions, and/or by the use of surfactants. Such formulations can be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations can be prepared, packaged, or sold in unit dosage form, such as in ampules, in multi-dose containers containing a preservative, or in single-use devices for auto-injection or injection by a medical practitioner.

Formulations for parenteral administration include suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations can further comprise one or more additional ingredients including suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for parenteral administration, the active ingredient is provided in dry (i.e. powder or granular) form for reconstitution with a suitable vehicle (e.g. sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition. The pharmaceutical compositions can be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution can be formulated according to the known art, and can comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations can be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butanediol, for example. Other acceptable diluents and solvents include Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer system. Compositions for sustained release or

implantation can comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

These compositions may also contain adjuvants such as preserving, wetting, emulsifying, and/or dispersing agents. Prevention of microorganism contamination of the compositions can be accomplished by the addition of various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of injectable pharmaceutical compositions can be brought about by the use of agents capable of delaying absorption, for example, aluminum monostearate and/or gelatin.

Dosage forms can include solid or injectable implants or depots. In preferred embodiments, the implant comprises an aliquot of the particulate delivery system and a biodegradable polymer. In preferred embodiments, a suitable biodegradable polymer can be selected from the group consisting of a polyaspartate, polyglutamate, poly(L-lactide), a poly(D,L-lactide), a poly(lactide-co-glycolide), a poly(ε-caprolactone), a polyanhydride, a poly(beta-hydroxy butyrate), a poly(ortho ester) and a polyphosphazene.

Solid dosage forms for oral administration include capsules, tablets, powders, and granules. In such solid dosage forms, the particulate delivery system is optionally admixed with at least one inert customary excipient (or carrier) such as sodium citrate or dicalcium phosphate or (a) fillers or extenders, as for example, starches, lactose, sucrose, mannitol, or silicic acid; (b) binders, as for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose, or acacia; (c) humectants, as for example, glycerol; (d) disintegrating agents, as for example, agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain complex silicates, or sodium carbonate; (e) solution retarders, as for example, paraffin; (f) absorption accelerators, as for example, quaternary ammonium compounds; (g) wetting agents, as for example, cetyl alcohol or glycerol monostearate; (h) adsorbents, as for example, kaolin or bentonite; and/or (i) lubricants, as for example, talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, or mixtures thereof. In the case of capsules and tablets, the dosage forms may also comprise buffering agents.

A tablet comprising the particulate delivery system can, for example, be made by compressing or molding the active ingredient, optionally with one or more additional ingredients. Compressed tablets can be prepared by compressing, in a suitable device, the active ingredient in a free-flowing form such as a powder or granular preparation, optionally mixed

with one or more of a binder, a lubricant, an excipient, a surface active agent, and a dispersing agent. Molded tablets can be made by molding, in a suitable device, a mixture of the active ingredient, a pharmaceutically acceptable carrier, and at least sufficient liquid to moisten the mixture. Pharmaceutically acceptable excipients used in the manufacture of tablets include inert diluents, granulating and disintegrating agents, binding agents, and lubricating agents. Known dispersing agents include potato starch and sodium starch glycolate. Known surface active agents include sodium lauryl sulfate. Known diluents include calcium carbonate, sodium carbonate, lactose, microcrystalline cellulose, calcium phosphate, calcium hydrogen phosphate, and sodium phosphate. Known granulating and disintegrating agents include corn starch and alginic acid. Known binding agents include gelatin, acacia, pre-gelatinized maize starch, polyvinylpyrrolidone, and hydroxypropyl methylcellulose. Known lubricating agents include magnesium stearate, stearic acid, silica, and talc.

Tablets can be non-coated or they can be coated using known methods to achieve delayed disintegration in the gastrointestinal tract of a human, thereby providing sustained release and absorption of the particulate delivery system, e.g. in the region of the Peyer's patches in the small intestine. By way of example, a material such as glyceryl monostearate or glyceryl distearate can be used to coat tablets. Further by way of example, tablets can be coated using methods described in U.S. Pat. Nos. 4,256,108; 4,160,452; and 4,265,874 to form osmotically-controlled release tablets. Tablets can further comprise a sweetening agent, a flavoring agent, a coloring agent, a preservative, or some combination of these in order to provide pharmaceutically elegant and palatable preparation.

Solid dosage forms such as tablets, dragees, capsules, and granules can be prepared with coatings or shells, such as enteric coatings and others well known in the art. They may also contain opacifying agents, and can also be of such composition that they release the particulate delivery system in a delayed manner. Examples of embedding compositions that can be used are polymeric substances and waxes. The active compounds can also be in micro-encapsulated form, if appropriate, with one or more of the above-mentioned excipients.

Solid compositions of a similar type may also be used as fillers in soft or hard filled gelatin capsules using such excipients as lactose or milk sugar, as well as high molecular weight polyethylene glycols, and the like. Hard capsules comprising the particulate delivery system can be made using a physiologically degradable composition, such as gelatin. Such hard capsules comprise the particulate delivery system, and can further comprise additional ingredients

including, for example, an inert solid diluent such as calcium carbonate, calcium phosphate, or kaolin. Soft gelatin capsules comprising the particulate delivery system can be made using a physiologically degradable composition, such as gelatin. Such soft capsules comprise the particulate delivery system, which can be mixed with water or an oil medium such as peanut oil, liquid paraffin, or olive oil.

Oral compositions can be made, using known technology, which specifically release orally-administered agents in the small or large intestines of a human patient. For example, formulations for delivery to the gastrointestinal system, including the colon, include enteric coated systems, based, e.g., on methacrylate copolymers such as poly(methacrylic acid, methyl methacrylate), which are only soluble at pH 6 and above, so that the polymer only begins to dissolve on entry into the small intestine. The site where such polymer formulations disintegrate is dependent on the rate of intestinal transit and the amount of polymer present. For example, a relatively thick polymer coating is used for delivery to the proximal colon (Hardy et al., 1987 Aliment. Pharmacol. Therap. 1:273-280). Polymers capable of providing site-specific colonic delivery can also be used, wherein the polymer relies on the bacterial flora of the large bowel to provide enzymatic degradation of the polymer coat and hence release of the drug. For example, azopolymers (U.S. Pat. No. 4,663,308), glycosides (Friend et al., 1984, J. Med. Chem. 27:261-268) and a variety of naturally available and modified polysaccharides (see PCT application PCT/GB89/00581) can be used in such formulations.

Pulsed release technology such as that described in U.S. Pat. No. 4,777,049 can also be used to administer the particulate delivery system to a specific location within the gastrointestinal tract. Such systems permit delivery at a predetermined time and can be used to deliver the particulate delivery system, optionally together with other additives that my alter the local microenvironment to promote stability and uptake, directly without relying on external conditions other than the presence of water to provide in vivo release.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs. In addition to the active compounds, the liquid dosage form may contain inert diluents commonly used in the art, such as water or other solvents, isotonic saline, solubilizing agents and emulsifiers, as for example, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils, in particular, almond oil, arachis oil, coconut oil, cottonseed oil, groundnut oil, corn germ oil, olive oil, castor oil, sesame seed oil,

MIGLYOL™, glycerol, fractionated vegetable oils, mineral oils such as liquid paraffin, tetrahydrofurfuryl alcohol, polyethylene glycols, fatty acid esters of sorbitan, or mixtures of these substances, and the like. Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, demulcents, preservatives, buffers, salts, sweetening, flavoring, coloring and perfuming agents. Suspensions, in addition to the active compound, may contain suspending agents, as for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol or sorbitan esters, microcrystalline cellulose, hydrogenated edible fats, sodium alginate, polyvinylpyrrolidone, gum tragacanth, gum acacia, agar-agar, and cellulose derivatives such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, aluminum metahydroxide, bentonite, or mixtures of these substances, and the like. Liquid formulations of a pharmaceutical composition of the invention that are suitable for oral administration can be prepared, packaged, and sold either in liquid form or in the form of a dry product intended for reconstitution with water or another suitable vehicle prior to use.

Known dispersing or wetting agents include naturally-occurring phosphatides such as lecithin, condensation products of an alkylene oxide with a fatty acid, with a long chain aliphatic alcohol, with a partial ester derived from a fatty acid and a hexitol, or with a partial ester derived from a fatty acid and a hexitol anhydride (e.g. polyoxyethylene stearate, heptadecaethyleneoxycetanol, polyoxyethylene sorbitol monooleate, and polyoxyethylene sorbitan monooleate, respectively). Known emulsifying agents include lecithin and acacia. Known preservatives include methyl, ethyl, or n-propyl-para-hydroxybenzoates, ascorbic acid, and sorbic acid. Known sweetening agents include, for example, glycerol, propylene glycol, sorbitol, sucrose, and saccharin. Known thickening agents for oily suspensions include, for example, beeswax, hard paraffin, and cetyl alcohol.

In other embodiments, the pharmaceutical composition can be prepared as a nutraceutical, i.e., in the form of, or added to, a food (e.g., a processed item intended for direct consumption) or a foodstuff (e.g., an edible ingredient intended for incorporation into a food prior to ingestion). Examples of suitable foods include candies such as lollipops, baked goods such as crackers, breads, cookies, and snack cakes, whole, pureed, or mashed fruits and vegetables, beverages, and processed meat products. Examples of suitable foodstuffs include milled grains and sugars, spices and other seasonings, and syrups. The particulate delivery

systems described herein are preferably not exposed to high cooking temperatures for extended periods of time, in order to minimize degradation of the compounds.

Compositions for rectal or vaginal administration can be prepared by mixing a particulate delivery system with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax, which are solid at ordinary room temperature, but liquid at body temperature, and therefore, melt in the rectum or vaginal cavity and release the particulate delivery system. Such a composition can be in the form of, for example, a suppository, a retention enema preparation, and a solution for rectal or colonic irrigation. Suppository formulations can further comprise various additional ingredients including antioxidants and preservatives. Retention enema preparations or solutions for rectal or colonic irrigation can be made by combining the active ingredient with a pharmaceutically acceptable liquid carrier. As is known in the art, enema preparations can be administered using, and can be packaged within, a delivery device adapted to the rectal anatomy of a human. Enema preparations can further comprise various additional ingredients including antioxidants and preservatives.

A pharmaceutical composition of the invention can be prepared, packaged, or sold in a formulation suitable for pulmonary administration via the buccal cavity. Such compositions are conveniently in the form of dry powders for administration using a device comprising a dry powder reservoir to which a stream of propellant can be directed to disperse the powder or using a self-propelling solvent/powder-dispensing container such as a device comprising the particulate delivery system suspended in a low-boiling propellant in a sealed container. Dry powder compositions may include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form. Low boiling propellants generally include liquid propellants having a boiling point below 65 degrees F. at atmospheric pressure. Generally the propellant can constitute 50 to 99.9% (w/w) of the composition, and the active ingredient can constitute 0.1 to 20% (w/w) of the composition. The propellant can further comprise additional ingredients such as a liquid non-ionic or solid anionic surfactant or a solid diluent (preferably having a particle size of the same order as particles comprising the particulate delivery system).

Pharmaceutical compositions of the invention formulated for pulmonary delivery can also provide the active ingredient in the form of droplets of a suspension. Such formulations can be prepared, packaged, or sold as aqueous or dilute alcoholic suspensions, optionally sterile, comprising the particulate delivery system, and can conveniently be administered using any

nebulization or atomization device. Such formulations can further comprise one or more additional ingredients including a flavoring agent such as saccharin sodium, a volatile oil, a buffering agent, a surface active agent, or a preservative such as methylhydroxybenzoate.

The formulations described herein as being useful for pulmonary delivery are also useful for intranasal delivery of a pharmaceutical composition of the invention. Another formulation suitable for intranasal administration is a coarse powder comprising the particulate delivery system. Such a formulation is administered in the manner in which snuff is taken i.e. by rapid inhalation through the nasal passage from a container of the powder held close to the nares.

A pharmaceutical composition of the invention can be prepared, packaged, or sold in a formulation suitable for buccal administration. Such formulations can, for example, be in the form of tablets or lozenges made using conventional methods, and can, for example, comprise 0.1 to 20% (w/w) particulate delivery system, the balance comprising an orally dissolvable or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for buccal administration can comprise a powder or an aerosolized or atomized solution or suspension comprising the particulate delivery system.

Antibodies

As used herein, the term "antibody" (Ab) or "monoclonal antibody" (Mab) is meant to include intact molecules as well as antibody fragments (such as, for example, Fab and F(ab')2 fragments) which are capable of specifically binding to protein. Fab and F(ab')2 fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody. Thus, these fragments are preferred, as well as the products of a Fab or other immunoglobulin expression library. Moreover, antibodies of the present invention include chimeric, single chain, and humanized antibodies.

Antibodies can be prepared using any number of techniques known in the art. Suitable techniques are discussed briefly below. The antibody may be polyclonal or monoclonal. Polyclonal antibodies can have significant advantages for initial development, including rapidity of production and specificity for multiple epitopes, ensuring strong immunofluorescent staining and antigen capture. Monoclonal antibodies are adaptable to large-scale production; preferred embodiments include at least one monoclonal antibody specific for an epitope of the target antigen. Because polyclonal preparations cannot be readily reproduced for large-scale production, another embodiment uses a cocktail of at least four monoclonal antibodies.

A single chain Fv ("scFv" or "sFv") polypeptide is a covalently linked V_H :V_L heterodimer which may be expressed from a nucleic acid including V_H - and V_L -encoding sequences either joined directly or joined by a peptide-encoding linker. Huston, et al. Proc. Nat. Acad. Sci. USA, 85: 5879-5883 (1988). A number of structures for converting the naturally aggregated, but chemically separated, light and heavy polypeptide chains from an antibody V region into a scFv molecule which folds into a three dimensional structure substantially similar to the structure of an antigen-binding site. See, e.g. U.S. Pat. Nos. 6,512,097, 5,091,513 and 5,132,405 and 4,956,778.

In one class of embodiments, recombinant design methods can be used to develop suitable chemical structures (linkers) for converting two naturally associated, but chemically separate, heavy and light polypeptide chains from an antibody variable region into a sFv molecule which folds into a three-dimensional structure that is substantially similar to native antibody structure. Design criteria include determination of the appropriate length to span the distance between the C-terminal of one chain and the N-terminal of the other, wherein the linker is generally formed from small hydrophilic amino acid residues that do not tend to coil or form secondary structures. Such methods have been described in the art. See, e.g., U.S. Pat. Nos. 5,091,513 and 5,132,405 to Huston et al.; and U.S. Pat. No. 4,946,778 to Ladner et al.

In this regard, the first general step of linker design involves identification of plausible sites to be linked. Appropriate linkage sites on each of the V_H and V_L polypeptide domains include those which result in the minimum loss of residues from the polypeptide domains, and which necessitate a linker comprising a minimum number of residues consistent with the need for molecule stability. A pair of sites defines a "gap" to be linked. Linkers connecting the C-terminus of one domain to the N-terminus of the next generally comprise hydrophilic amino acids which assume an unstructured configuration in physiological solutions and preferably are free of residues having large side groups which might interfere with proper folding of the V_H and V_L chains. Thus, suitable linkers under the invention generally comprise polypeptide chains of alternating sets of glycine and serine residues, and may include glutamic acid and lysine residues inserted to enhance solubility. Nucleotide sequences encoding such linker moieties can be readily provided using various oligonucleotide synthesis techniques known in the art.

Alternatively, a humanized antibody fragment may comprise the antigen binding site of a murine monoclonal antibody and a variable region fragment (lacking the antigen binding site) derived from a human antibody. Procedures for the production of chimeric and further

engineered monoclonal antibodies include those described in Riechmann et al. (Nature 332 : 323,1988), Liu et al. (PNAS 84 : 3439,1987), Larrick et al. (Bio Technology 7: 934,1989), and Winter and Harris (TIPS 14 : 139, May, 1993).

One method for producing a human antibody comprises immunizing a nonhuman animal, such as a transgenic mouse, with a target antigen, whereby antibodies directed against the target antigen are generated in said animal. Procedures have been developed for generating human antibodies in non-human animals. The antibodies may be partially human, or preferably completely human. Non-human animals (such as transgenic mice) into which genetic material encoding one or more human immunoglobulin chains has been introduced may be employed. Such transgenic mice may be genetically altered in a variety of ways. The genetic manipulation may result in human immunoglobulin polypeptide chains replacing endogenous immunoglobulin chains in at least some (preferably virtually all) antibodies produced by the animal upon immunization. Antibodies produced by immunizing transgenic animals with a target antigen are provided herein.

Mice in which one or more endogenous immunoglobulin genes are inactivated by various means have been prepared. Human immunoglobulin genes have been introduced into the mice to replace the inactivated mouse genes. Antibodies produced in the animals incorporate human immunoglobulin polypeptide chains encoded by the human genetic material introduced into the animal. Examples of techniques for production and use of such transgenic animals are described in U. S. Patents 5,814,318, 5,569,825, and 5,545,806, which are incorporated by reference herein.

Monoclonal antibodies may be produced by conventional procedures, e. g., by immortalizing spleen cells harvested from the transgenic animal after completion of the immunization schedule. The spleen cells may be fused with myeloma cells to produce hybridomas, by conventional procedures.

A method for producing a hybridoma cell line comprises immunizing such a transgenic animal with a immunogen comprising at least seven contiguous amino acid residues of a target antigen; harvesting spleen cells from the immunized animal; fusing the harvested spleen cells to a myeloma cell line, thereby generating hybridoma cells; and identifying a hybridoma cell line that produces a monoclonal antibody that binds a target antigen. Such hybridoma cell lines, and monoclonal antibodies produced therefrom, are encompassed by the present invention.

Monoclonal antibodies secreted by the hybridoma cell line are purified by conventional techniques.

In another embodiment, antibody fragments are produced by selection from a nonimmune phage display antibody repertoire against one set of antigens in the presence of a competing set of antigens (Stausbol-Grøn, B., et al., *De novo* identification of cell-type specific antibody-antigen pairs by phage display subtraction. Isolation of a human single chain antibody fragment against human keratin 14. Eur J Biochem 2001 May; 268(10):3099-107). This approach can be used to produce phage antibodies directed against target antigens. The protocol in general is based on that described by Stausbol-Grøn, B., et al., 2001. Briefly, a nonimmunized semisynthetic phage display antibody repertoire is used. The repertoire is a single chain Fv (scFv) phagemid repertoire constructed by recloning the heavy and light chain regions from the lox library (Griffiths, A.D., et al. (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J. 13, 3245-3260.). *Escherichia coli* TG1 (*supE* hsdD5 Δ(*lac-proAB*) *thi* F'{*traD*36 *pro*AB+ *lacI*^{q} *lac*ZΔM15]) is an amber suppressor strain (*supE*) and is used for propagation of phage particles. *E. coli* HB2151 (*ara* Δ(*lac-proAB*) *thi* F'{*pro*AB+ *lacI*^{q} *lac*ZΔM15]) is a nonsuppressor strain and is used for expression of soluble scFv. In another embodiment, a human single-chain Fv (scFv) library can be amplified and rescued, as described (Gao, at al., Making chemistry selectable by linking it to infectivity, Proc. Natl. Acad. Sci. USA, Vol. 94, pp. 11777-11782, October 1997). The library is panned against target antigens suspended in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) and the positive scFv-phage are selected by enzyme-linked immunosorbent assay (ELISA).

In other preferred embodiments, an antibody is supplied by providing an expression vector encoding a recombinant antibody, preferably a single chain Fv antibody.

Example 1

Figure 1 is a schematic diagram 100 of a transverse section of a yeast cell wall, showing, from outside to inside, an outer fibrillar layer 110, an outer mannoprotein layer 120, a beta glucan layer 130, a beta glucan layer – chitin layer 140, an inner mannoprotein layer 150, the plasma membrane 160 and the cytoplasm 170.

Preparation of WGP Particles

Whole Glucan Particles (WGP, Lot W0282) were previously obtained from Alpha-Beta Technology. In general, whole glucan particles are prepared from yeast cells by the extraction

and purification of the alkali-insoluble glucan fraction from the yeast cell walls. The yeast cells are treated with an aqueous hydroxide solution without disrupting the yeast cell walls, which digests the protein and intracellular portion of the cell, leaving the glucan wall component devoid of significant protein contamination, and having substantially the unaltered cell wall structure of β(1-6) and β(1-3) linked glucans. Yeast cells (*S. cerevisae* strain R4) were grown to midlog phase in minimal media under fed batch fermentation conditions. Cells (~90 g dry cell weight/L) were harvested by batch centrifugation at 2000 rpm for 10 minutes. The cells were then washed once in distilled water and then resuspended in 1 liter of 1M NaOH and heated to 90 degrees Celsius. The cell suspension was stirred vigorously for 1 hour at this temperature. The insoluble material, containing the cell walls, was recovered by centrifuging at 2000 rpm for 10 minutes. This material was then suspended in 1 liter, 1M NaOH and heated again to 90 degrees Celsius. The suspension was stirred vigorously for 1 hour at this temperature. The suspension was then allowed to cool to room temperature and the extraction was continued for a further 16 hours. The insoluble residue was recovered by centrifugation at 2000 rpm for 10 minutes. This material was finally extracted in 1 liter, water brought to pH 4.5 with HCl, at 75 degrees Celsius for 1 hour. The insoluble residue was recovered by centrifugation and washed three times with 200 milliliters water, four times with 200 milliliters isopropanol and twice with 200 milliliters acetone. The resulting slurry was placed in glass trays and dried at 55 degrees Celsius under reduced pressure to produce 7.7 g of a fine white powder.

A more detailed description of whole glucan particles and a process of preparing them can be found in U.S. Pats. Nos. 4,810,646; 4,992,540; 5,028,703; 5,607,677 and 5,741,495, the teachings of which are incorporated herein by reference. For example, U.S. Pat. No. 5,028,703 discloses that yeast WGP particles can be produced from yeast cells in fermentation culture. The cells were harvested by batch centrifugation at 8000 rpm for 20 minutes in a Sorval RC2-B centrifuge. The cells were then washed twice in distilled water in order to prepare them for the extraction of the whole glucan. The first step involved resuspending the cell mass in 1 liter 4% w/v NaOH and heating to 100 degrees Celsius. The cell suspension was stirred vigorously for 1 hour at this temperature. The insoluble material containing the cell walls was recovered by centrifuging at 2000 rpm for 15 minutes. This material was then suspended in 2 liters, 3% w/v NaOH and heated to 75 degrees Celsius. The suspension was stirred vigorously for 3 hours at this temperature. The suspension was then allowed to cool to room temperature and the extraction was continued for a further 16 hours. The insoluble residue was recovered by

centrifugation at 2000 rpm for 15 minutes. This material was finally extracted in 2 liters, 3% w/v NaOH brought to pH 4.5 with HCl, at 75 degrees Celsius for 1 hour. The insoluble residue was recovered by centrifugation and washed three times with 200 milliliters water, once with 200 milliliters dehydrated ethanol and twice with 200 milliliters dehydrated ethyl ether. The resulting slurry was placed on petri plates and dried.

Preparation of YGMP Particles

S. cerevisiae (100 g Fleishmans Bakers yeast) was suspended in 1 liter 1M NaOH and heated to 55 degrees Celsius. The cell suspension was mixed for 1 hour at this temperature. The insoluble material containing the cell walls was recovered by centrifuging at 2000 rpm for 10 minutes. This material was then suspended in 1 liter of water and brought to pH 4-5 with HCl, and incubated at 55 degrees Celsius for 1 hour. The insoluble residue was recovered by centrifugation and washed once with 1000 milliliters water, four times with 200 milliliters dehydrated isopropanol and twice with 200 milliliters acetone. The resulting slurry was placed in a glass tray and dried at room temperature to produce 12.4 g of a fine, slightly off-white, powder.

Preparation of YGMP Particles

S. cerevisiae (75 g SAF-Mannan) was suspended in 1 liter water and adjusted to pH 12-12.5 with 1M NaOH and heated to 55 degrees Celsius. The cell suspension was mixed for 1 hour at this temperature. The insoluble material containing the cell walls was recovered by centrifuging at 2000 rpm for 10 minutes. This material was then suspended in 1 liter of water and brought to pH 4-5 with HCl, and incubated at 55 degrees Celsius for 1 hour. The insoluble residue was recovered by centrifugation and washed once with 1000 milliliters water, four times with 200 milliliters dehydrated isopropanol and twice with 200 milliliters acetone. The resulting slurry was placed in a glass tray and dried at room temperature to produce 15.6 g of a fine slightly off-white powder.

Preparation of YCP Particles

Yeast cells (*Rhodotorula* sp.) derived from cultures obtained from the American Type Culture Collection (ATCC, Manassas, VA) were aerobically grown to stationary phase in YPD at 30 degrees Celsius. *Rhodotorula* sp. cultures available from ATCC include Nos. 886, 917, 9336, 18101, 20254, 20837 and 28983. Cells (1L) were harvested by batch centrifugation at

2000 rpm for 10 minutes. The cells were then washed once in distilled water and then resuspended in water brought to pH 4.5 with HCl, at 75 degrees Celsius for 1 hour. The insoluble material containing the cell walls was recovered by centrifuging at 2000 rpm for 10 minutes. This material was then suspended in 1 liter, 1M NaOH and heated to 90 degrees Celsius for 1 hour. The suspension was then allowed to cool to room temperature and the extraction was continued for a further 16 hours. The insoluble residue was recovered by centrifugation at 2000 rpm for 15 minutes and washed twice with 1000 milliliters water, four times with 200 milliliters isopropanol and twice with 200 milliliters acetone. The resulting slurry was placed in glass trays and dried at room temperature to produce 2.7 g of a fine light brown powder.

Figure 2A is a diagram of the structure of a yeast cell wall particle; Figure 2B is a fluorescence photomicrograph showing concanavalin-A-FITC (con-A-fluorescein isothiocyanate, Sigma Chemical, St. Louis, MO) staining of the mannan component of the yeast cell wall particles; Figure 2C is a diagram of the structure of a YGMP beta glucan-mannan particle, Figure 2D is a fluorescence photomicrograph showing punctuate con-A-FITC staining of a YGMP beta glucan-mannan particle; Figure 2E is a diagram of the structure of a YGP beta glucan particle and Figure 2F is a fluorescence micrograph showing the absence of con-A-FITC staining of a YGP beta glucan particle.

Concanavalin-A is a lectin that binds selectively to mannose. Concanavalin-A-FITC binding was evaluated by fluorescence microscopy in order to observe the amount and distribution pattern of mannan on the surface of various yeast cell wall preparations. Suspensions of Baker's yeast (Fleishmans Bakers yeast), YGMP and YGP in PBS + 1mM $MgCl_2$ +1mM $CaCl_2$ were prepared at a density of 1 x 10^8 particles/ml. Con-A-FITC stock was 1 mg/ml concanavalin-A-FITC in PBS + 1mM $MgCl_2$ +1mM $CaCl_2$. Labeling mixtures were prepared in microcentrifuge tubes consisting of:

100 µl PBS + 1mM $MgCl_2$ +1mM $CaCl_2$

2.5 µl yeast cell wall particle suspension

2.5 µl con-A-FITC stock solution.

The microcentrifuge tubes containing the labeling mixtures were incubated in the dark at room temperature for one hour. Yeast cell wall particles were collected by centrifugation (10,000 rpm for 10 minutes) followed by washing the pellet with 100 µl PBS three times. The washed yeast cell wall particles were resuspended in 100 µl PBS and transferred to a 96 well plate for

examination with a fluorescence microscope. Photographs of exemplary fields are shown in Figures 2B, 2D and 2F.

Table 1 summarizes the results of analyses of the chemical composition of WGP particles, YGP particles, YGMP particles and YCP particles that were prepared as described above. Note that YGP particles and YGMP particles have lower beta-glucan and higher protein compared to the prior art WGP particles. YGMP particles have a substantially higher mannan content compared to the other particle types. YCP particles have a substantially higher chitin + chitosan content compared to the other particle types.

Table 1

Chemical Composition of Yeast Cell Wall Materials

Analyte	**Method**	**WGP** *S. cerevisiae*	**YGMP** *S. cerevisiae*	**YGP** *S. cerevisiae*	**YCP** *Rhodotorula*
Macromolecular Composition*					
Protein	Kjeldal	<1	4.5	4.9	-
Fat	Base hydrolysis, Soxhlet extraction	<1	1.6	1.4	-
Ash	Combustion	1.2	1.9	1.6	-
Carbohydrate Composition**					
Beta-Glucan	Enzymatic Hydrolysis	90.3	41.9	77	6.5
Chitin + chitosan (as glucosamine, n-acetyl glucosamine)	Monosac Analysis-Dionex	2.1	2.3	2.4	68
Mannan (as mannose)	Monosac Analysis-Dionex	<1	36.9	0.47	1.3
Other Glucans (as non beta 1,3-glucose and other unmeasured sugars)	Monosac Analysis-Dionex	6.2	10.9	11.2	0.2

*Results are reported % w/w of dry analyzed materials
**Results are reported % w/w carbohydrate

WGP – Whole Glucan Particle – Prior Art Technology
YGMP – Yeast Glucan-Mannan Particle
YGP – Yeast Glucan Particle
YCP –Yeast Chitin Particle

Example 2 Hydrodynamic Volume of Yeast Cell Wall Particles

The hydrodynamic volume of yeast cell wall particles was determined as a measure of the payload capacity of the particles. A 1 g aliquot of yeast cell wall particles was weighed in a tared 15 ml centrifuge tube to determine the weight of the dry particles. Water (12.5 ml) was added to the tube, and the tube was vortexed to mix the suspension of yeast cell wall particles. The particles were allowed to swell and absorb water for 30 minutes. The particle suspension was centrifuged at 2000 rpm for 10 minutes. The water was removed, the tube was weighed, and the weight of water absorbed was calculated. The hydrodynamic volume was calculated as the ratio of the weight of the water absorbed to the weight of the dry particles. Table 2 presents the results for two preparations of the prior art WGP and the YGP and YGMP of the present invention.

Table 2

Hydrodynamic Volume of Exemplary Yeast Cell Wall Preparations

Yeast Cell Wall Particle	Hydrodynamic Volume (g water/g particles)
WGP Prep 1	9.7
WGP Prep 2	6.9
YGP	8.3
YGMP	6.7

The lower hydrodynamic volume of WGP Prep 2 may be due to an increased number of fragmented particles in this preparation. With respect to the other particles, the "purer" YGP had a higher hydrodynamic volume than the YGMP.

In general, the payload volume was limited to <66% hydrodynamic volume to ensure quantitative absorption of the payload by the yeast cell wall particles. By this rule, ≤5.5 µl payload would be loaded per mg YGP particles and ≤4.4 µl payload would be loaded per mg YGMP particles.

Example 3 Oral Bioavailability of YGP and YGMP

Fluorescently labeled yeast glucan particles (YGP-F) and fluorescently labeled yeast glucan-mannan particles (YGMP-F) were prepared for an uptake study. Starting materials were: 5 ml YGP (5 mg/ml in 0.1M borate buffer, pH 8), 5 ml YGMP (5 mg/ml in 0.1M borate buffer, pH8), dichlorotriazinyl aminofluorescein (DTAF), 20 mg/ml in DMSO, freshly prepared and 0.1M borate buffer, pH 8.

Labeling reactions were carried out at a 25 mg scale. Aliquots of 25 mg particles were suspended in 5 ml 0.1M borate buffer, pH 8 and sonicated to reduce clumps of particles to single particles. The particles were centrifuged and resuspended in 5 ml 0.1M borate buffer, pH 8. DTAF (0.5 ml 20 mg/ml) was added to the resuspended particles and incubated 2 days at 37 degrees Celsius. At the end of the incubation, 5 ml 1 M Tris buffer, pH 8.3, was added and the mixture was incubated 30 minutes to quench DTAF. The incubated particles were centrifuged and washed in PBS until the supernatants were no longer fluorescent. The washed particles were resuspended in PBS at 5 mg/ml. The number of particles in a 1:100 dilution of an aliquot was counted. Results: intensely fluorescent yeast cell wall particles were produced, at concentrations of 1.8×10^9 particles per ml YGP-F and 2.1×10^9 particles per ml YGMP-F.

The influence of the surface carbohydrate composition on the oral bioavailability of yeast glucan particles was studied to determine if the phagocytic particle uptake of a payload could be targeted via the mannose receptor as well as by the CR3/dectin-1 beta glucan receptors. The ability to target either or both receptors can expand the target population of cells beyond macrophages and dendritic cells.

The treatment groups are summarized in Table 3, below. Starting materials included: FITC-labeled yeast glucan particles (YGP-F), FITC-labeled yeast glucan-mannan particles (YGMP-F), a group of seven C57Black mice and a group of seven C57/B16 mice. Doses of YGP-F (1 mg/ml) and YGMP-F (3.7 mg/ml) were prepared to deliver equivalent number of particles in 0.1 ml PBS and administered by oral gavage to one mouse from each group daily for five days. The same dose was administered by subcutaneous injection of 0.1 ml to one mouse from each group daily for five days. On day four the cages were changed and fresh bedding was provided. Fecal pellets were collected on day 5 from each group into 15 ml conical tubes and frozen for processing later. The fecal pellets were processed by adding 5 ml water and holding at 4 degrees Celsius for 2 hours. The hydrated fecal pellets were homogenized using a Polytron homogenizer. Dilutions of homogenized feces were placed in a 96-well microtiter plate and microscopically examined under fluorescent and transmitted white light conditions for the presence of fluorescent particles. Aliquots having fluorescent particles were further diluted and the number of fluorescent particles/ml was counted with a hematocytometer.

Mice were sacrificed on day 7, and the spleen was removed from each animal and placed into separate tubes containing PBS on ice. The spleens were macerated with scissors and pressed through 70 micron screens to produce single cell suspensions. Aliquots of the single cell

suspensions were retained and fixed in 1% formalin in PBS for quantifying the fraction of cells labeled with fluorescent particles using FACS. Cell suspensions are stained using a phycoerythrin (PE) labeled-antibody against macrophage marker, preferably murine Emr-1 (F4/80), which stains splenic red pulp macrophages, Kupffer cells, microglia and Langerhans cells.

Cell suspensions were plated at a density of 10^7 cells per 60mm petri dish in DMEM containing 10% fetal calf serum (JRH Scientific), penicillin-streptomycin and glutamine (Gibco) and incubated for 24 hours at 37 degrees Celsius under 5% CO_2 to allow for attachment. After the incubation, any unattached lymphocytes were washed away. The attached splenic macrophage cells were typsinized, fixed and scored for the fraction of adherent cells having fluorescent particles using a fluorescence microscope.

The administration of the fluorescent particles was well tolerated. Analysis of adherent splenic macrophages demonstrated the presence of fluorescent yeast cell wall particles in all fluorescent particle treated animals. These results demonstrate that both YGP-F and YGMP-F are orally bioavailable and can be systemically distributed by macrophages. The analysis of feces demonstrated the presence of fluorescent particles, indicating that oral absorption was incomplete at the dosage levels used. C57/B16 mice were able to absorb YGP-F and YGMP-F administered orally. The number of fluorescent particles in feces was quantified as an estimate of uptake efficiency.

Table 3

						Presence of Fluorescent Particles	
Route	**Treatment**	**Dose**	**mg/ml**	**# part./ml**	**# part./dose**	**Splenic Macrophages**	**Feces**
	Control	PBS control	-	-	-		
SQ	YGP-F	100 µg	1	$1x10^9$	$1x10^8$	+	-
Oral	YGP-F	100 µg	1	$1x10^9$	$1x10^8$	+	+
Oral	YGP-F	33 µg	0.33	$3.3x10^8$	$3.3x10^7$	+	+
SQ	YGPM-F	370 µg	3.7	$1x10^9$	$1x10^8$	+	-
Oral	YGPM-F	370 µg	3.7	$1x10^9$	$1x10^8$	+	+
Oral	YGPM-F	110 µg	1.1	$3.3x10^8$	$3.3x10^7$	+	+
Untreated Control	-	-	-	-	-	-	-

Example 4: Preparation of Chitosan Loaded YGP Particles

YGP particles were prepared with a cationic trapping polymer, chitosan. 1% w/v chitosan solutions were prepared in 0.1M acetic acid using either High Molecular Weight (HMW) chitosan (~ 70,000 Mw, Sigma Chemical St. Louis, Mo) or Low Molecular Weight (HMW) chitosan (~ 10,000 Mw, Sigma Chemical St. Louis, Mo). Both 1% w/v HMW and LMW chitosan solutions were prepared in 0.1M acetic acid. Four ml HMW or LMW chitosan solution was added to 2 g YGP in a 50 ml conical centrifuge tube and mixed until a smooth paste was formed. The mixture was incubated for 1 hour at room temperature to allow the liquid to be absorbed. NaOH (40 ml, 0.1M) was added to each tube, which was vortexed immediately to precipitate the chitosan inside the YGP. The YGP:chitosan suspension was passed through an 18 gauge needle to produce a fine suspension of YGP:chitosan particles. The YGP:chitosan particles were collected by centrifugation (2,000 rpm for 10 minutes) followed by washing the pellet with deionized water until the pH of the supernatant was 7-8. The YGP:chitosan particles were then washed four times with two pellet volumes of isopropanol and then washed twice with two pellet volumes of acetone. The YGP:chitosan particles were then dried at room temperature in a hood. The procedure yielded 1.2 g YGP:LMW chitosan particles and 1.4 g YGP:HMW chitosan particles.

Example 5: Preparation of CytoPure™ Loaded YGP Particles

YGP particles were prepared with a biodegradable cationic trapping polymer, CytoPure™, a proprietary, commercially available, water-soluble cationic polymer transfection reagent (Qbiogene, Inc., CA). Twenty µl CytoPure™ was diluted in 0.5 ml deionized water and added to 0.5 g YGP in a 50 ml conical centrifuge tube and mixed until a smooth paste was formed. The mixture was incubated for 15 minutes at 4 degrees Celsius to allow the liquid to be absorbed. Twenty-five ml ethanol was added to each tube, which was vortexed immediately to precipitate the CytoPure™ inside the YGP. The YGP:CytoPure™ suspension was sonicated to produce a fine suspension of YGP:CytoPure™ particles. The YGP:CytoPure™ particles were collected by centrifugation (2,000 rpm for 10 minutes) followed by washing the pellet four times with two pellet volumes of isopropanol and then washed twice with two pellet volumes of acetone. The YGP:CytoPure™ particles were then dried at room temperature in a hood. The procedure yielded 0.45 g YGP:CytoPure™ particles.

Example 6: Preparation of Polyethylenimine Loaded YGP Particles

YGP particles were prepared with polyethylenimine (PEI) as a cationic trapping polymer. A 0.5 ml aliquot of a 2% w/v PEI (~ 50,000 Mw, Sigma Chemical Co., St. Louis, MO) solution in water was added to 0.5 g YGP in a 50 ml conical centrifuge tube and mixed until a smooth paste was formed. The mixture was incubated for one hour at room temperature to allow the liquid to be absorbed. Twenty-five ml ethanol was added to each tube, which was vortexed immediately to precipitate the PEI inside the YGP. The YGP:PEI suspension was passed through an 18 gauge needle to produce a fine suspension of YGP:PEI particles. The YGP:PEI particles were collected by centrifugation (2,000 rpm for 10 minutes) followed by washing the pellet four times with two pellet volumes of isopropanol and then washed twice with two pellet volumes of acetone. The YGP:PEI particles were then dried at room temperature in a hood. The procedure yielded 0.48 g YGP:PEI particles.

Example 7: Preparation of Alginate Loaded YGP Particles

YGP particles were prepared with alginate (F200 or F200L, Multi-Kem Corp., Ridgefield, NJ) as an anionic trapping polymer. A 2 ml aliquot of a 1% w/v alginate solution in water was added to 1 g YGP in a 50 ml conical centrifuge tube and mixed to form a smooth paste. The mixture was incubated for one hour at room temperature to allow the liquid to be absorbed. The mixture was diluted with 40 ml of a 1% w/v calcium chloride aqueous solution. The YGP:alginate suspension was passed through an 18 gauge needle to produce a fine suspension of YGP:alginate particles. The YGP:alginate particles were collected by centrifugation (2,000 rpm for 10 minutes. The YGP:alginate particles were washed four times with two pellet volumes of isopropanol and then washed twice with two pellet volumes of acetone. The YGP:alginate particles were then dried at room temperature in a hood. The procedure yielded 0.95 g YGP:F200 alginate particles and 0.86 g YGP:F200L alginate particles.

Example 8: Preparation of Poly-L-lysine Loaded YGP and YGMP Particles

YGP and YGMP particles were prepared with Poly-L-lysine (PLL) as a trapping polymer. A 4 ml aliquot of a 1% w/v PLL (Sigma Chemical Co., St. Louis, MO) solution in water was added to 1 g YGP or YGMP in a 50 ml conical centrifuge tube. The mixture was incubated for 30 minutes at 55 degrees Celsius to allow the liquid to be absorbed. Ten ml ethanol was added to each tube, which was homogenized (Polytron homogenizer) to produce a fine suspension of

YGP:PLL or YGMP:PLL particles. The YGP:PLL or YGMP:PLL particles were collected by centrifugation (2,000 rpm for 10 minutes. The YGP:PLL or YGMP:PLL were washed four times with two pellet volumes of isopropanol and then washed twice with two pellet volumes of acetone. The YGP:PLL or YGMP:PLL particles were then dried at room temperature in a hood. The procedure yielded 1.3 g YGP:PLL particles and 1.1 g YGMP:PLL particles. Microscopic evaluation showed no free PLL aggregates, only YGP:PLL or YGMP:PLL particles.

Example 9: Preparation of Xanthan Loaded YGP and YGMP Particles

YGP and YGMP particles were prepared with xanthan as an anionic trapping polymer. A 4 ml aliquot of a 1% w/v xanthan solution in water was heated to 55 degrees Celsius to reduce viscosity and added to 1 g YGP or YGMP in a 50 ml conical centrifuge tube. The mixture was incubated for 30 minutes at 55 degrees Celsius. Ten ml ethanol was added to each tube, which was homogenized (Polytron homogenizer) to produce a fine suspension of YGP:xanthan or YGMP:xanthan particles. The YGP:xanthan or YGMP:xanthan particles were collected by centrifugation (2,000 rpm for 10 minutes). The YGP:xanthan or YGMP:xanthan particles were washed four times with two pellet volumes of isopropanol and then washed twice with two pellet volumes of acetone. The YGP:xanthan or YGMP:xanthan particles were then dried at room temperature in a hood. The procedure yielded 1.2 g YGP:xanthan particles and 1.1 g YGMP:xanthan particles. Microscopic evaluation showed no free xanthan aggregates, only YGP:xanthan or YGMP:xanthan particles.

Example 10: Evaluation of Ability of YGP:Chitosan and YGP:Alginate To Bind Charged Dyes

YGP:Chitosan and YGP:Alginate particles were prepared as described in Examples 7 & 9 above. 0.1% w/v aqueous solutions of trypan blue (Benzamine blue; CI 23850), an anionic dye and xylene cyanol (acid blue, a cationic dye) were prepared. A 50 µl aliquot of a 0.1% w/v aqueous dye solution was added to 10 mg YGP, YGP:Chitosan or YGP:Alginate in microcentrifuge tubes and the mixture was incubated for 1 hour at room temperature. The pellets were washed with deionized water until the supernatant solutions were no longer colored. The color of the pellet was evaluated; the results are presented in Table 4, below.

Table 4		
	Pellet Color	
YGP Formulation	Trypan blue	Xylene cyanol
YGP	Tan	Tan
YGP: Chitosan	Blue	Tan
YGP: Alginate	Tan	Green

Electrostatic interactions between insoluble trapping polymers inside YGP were capable of binding to oppositely charged low molecular weight model dye payloads.

Example 11: Use of YGP:Agarose to Trap Molecules by Physical Entrapment

YGP:Agarose was prepared to evaluate physical entrapment as a means to trap a payload in YGP. A 2% w/v solution of agarose (Sigma Chemical Co., St. Louis, MO) was prepared in TE, and cooled to 50 degrees Celsius. A 1 mg/ml stock solution of salmon sperm DNA in TE was diluted to 0.5 mg/ml DNA in TE or in 1% agarose at 50 degrees Celsius. A 500 mg aliquot of YGP was mixed with 500 µl of DNA in TE or 500 µl of DNA in agarose at 50 degrees Celsius and the mixture was incubated 1 hour at 50 degrees Celsius. The mixture was then cooled for 1 hour in a refrigerator to solidify the agarose. After 1 hour, 10 mls of TE was added and the mixture was incubated overnight in refrigerator. The mixture was then centrifuged, and DNA in the supernatant was measured by absorption at 260 nm. About >80% of the applied DNA was retained by YGP:Agarose compared to <1% retained by the YGP:TE control. These results indicate that agarose effectively traps DNA inside YGP by physical entrapment.

Example 12: Use of YGP:Polyacrylamide to Trap Molecules by Physical Entrapment

YGP:Polyacrylamide was prepared to evaluate physical entrapment as a means to trap a payload in YGP. A 1mg/ml stock solution of salmon sperm DNA in TE was diluted to 0.5 mg/ml DNA in TE or in 30% polyacrylamide/bis (Sigma Chemical Co., St. Louis, MO). TEMED (N,N,N',N'-Tetramethylethylenediamine) was added to each DNA mixture (1 µl TEMED to 5 mls of DNA solution), and a 2 ml aliquot of each solution was added to 1 g YGP. The result was mixed to form a uniform suspension and incubated 3 hours at room temperature. After the 3 hour incubation, 10 ml of TE was added and the mixture was incubated overnight in a refrigerator. The mixture was then centrifuged, and DNA in the supernatant was measured by absorption at 260 nm. About >95% of the applied DNA was retained by YGP:Polyacrylamide

compared to <1% retained by the YGP:TE control. These results indicate that polyacrylamide is an effective trapping polymer to use to trap DNA inside YGP by physical entrapment.

Example 13: Loading YGP With A Small Molecule, Tetracycline

The antibiotic tetracycline (tet) was loaded into YGP using the relative insolubility of the tetracycline-calcium salt. Yeast cell wall particles used were YGP, YGP:F200 alginate and YGP: F200L alginate prepared as described above. Stock solutions were 1 M $CaCl_2$ and 100 mg/ml tetracycline HCl (Sigma Chemical Co., St. Louis, MO). The loading mixtures were set up as summarized in Table 5, below.

Table 5

				Loading			Release	
				A355*	% tet	% tet	A355	
YGP (1 mg)	Tet (µl)	Water (µl)	1M $CaCl_2$ (µl)	super	bound	w/w	PBS	0.1M HCl
-	-	-	200	0	-	-	-	-
-	4	200	-	0.538	-	-	-	-
-	4	-	200	0.542	-	-	-	-
YGP	-	-	200	0.01	-	-	-	-
YGP	4	200	-	0.56	0	-	-	-
YGP	4	-	200	0.524	<1	-	-	-
YGP-F200 alginate	4	200	-	0.405	24.8	9.9	3.6	4.9
YGP-F200L alginate	4	200	-	0.375	30.3	12.1	5.9	12.2
				*1/100 dilution				

The mixtures were incubated for 30 minutes at room temperature and then deionized water or 1 M $CaCl_2$ was added as indicated. After 60 minutes at room temperature, the mixtures were sonicated and were incubated for at least an additional 30 minutes at room temperature. The mixtures were then centrifuged (2,000 rpm for 10 minutes) and the presence of tetracycline was indicated by the yellow color of the pellet and that of the initial supernatant. The amount of tetracycline loading into the yeast cell wall particles was calculated from the loss of absorption at 355 nm, the peak of the tetracycline absorption spectrum. A dilution of 4 µl of the 100 mg/ml tetracycline HCl stock solution in 200 µl deionized water had an absorbance at 355 nm of 0.538 compared to a deionized water blank. Release of tetracycline from the loaded yeast cell wall particles into PBS or 0.1M HCl was also measured spectrophotometrically.

The results are summarized in Table 5, above. In general, while YGP:F200 alginate and YGP:F200L alginate pellets were yellow after washing, YGP pellets were not yellow, indicating

little, if any, tetracycline loading either as the hydrochloride or the calcium salt in the absence of a trapping polymer. In contrast, tetracycline was effectively loaded and trapped in YGP:F200 alginate and YGP:F200L alginate formulations, with about 25-30% of the applied tetracycline load absorbed as the calcium alginate salt. Trapped tetracycline was released from YGP:F200 alginate and YGP:F200L alginate into 0.1M HCl. The trapped tetracycline was partially retained in YGP:F200 alginate and YGP:F200L alginate in PBS for 1 hour at 37 degrees Celsius, about 26.5 - 51.6% of 0.1M HCl extractable.

In summary, tetracycline was readily trapped as a calcium alginate salt complex in a YGP-alginate-calcium composition, but was not effectively loaded and retained within YGP alone. The tetracycline trapped as a calcium alginate complex in YGP:F200 alginate and YGP:F200L alginate was slowly released in PBS at 37 degrees Celsius and substantially released under acid conditions.

Example 14: Efficacy of Tet and YGP:Tet In Increasing *in vitro* Microbiocidal Killing of J774 Macrophages

YGP: alginate – tet was prepared as described in Example 13, above. The numbers of particles of YGP and YGP: alginate – tet per ml in the stock solutions were 9×10^7/ml and 6×10^8/ml, respectively.

Table 6

***S.aureus* Killing By J774 Murine Macrophages Loaded With YGP Particles**

YGP Tube	J774 5×10^5/ml	DMEM+C	YGP/tet 5×10^7/ml	µl	Particles/ml	*S. aureus* Killed	Fold Increased Killing
a	1 ml	0.1 ml	-	-	-	$< 1 \times 10^5$	1
b	-	1.1 ml	-	-	-	$< 1 \times 10^5$	1
c	1 ml	-	YGP	100	3×10^7	$< 1 \times 10^5$	1
d	-	1 ml	YGP	100	3×10^7	$< 1 \times 10^5$	1
e	1 ml	-	YGP:tet	100	3.75×10^6	1×10^8	100
f	-	1 ml	YGP:tet	100	3.75×10^6	1×10^6	-
g	1 ml	-	YGP:tet	100	7.5×10^6	$>1 \times 10^8$	>10
h	-	1 ml	YGP:tet	100	7.5×10^6	1×10^7	-
i	1 ml	-	YGP:tet	100	1.5×10^7	$>1 \times 10^8$	-
j	-	1 ml	YGP:tet	100	1.5×10^7	$>1 \times 10^8$	-
k	1 ml	-	YGP:tet	100	3×10^7	$>1 \times 10^8$	-
l	-	1 ml	YGP:tet	100	3×10^7	$>1 \times 10^8$	-
m	1 ml	-	tet - 1.25	100	1.25 µg/ml	1×10^6	-
n	-	1 ml	tet - 1.25	100	1.25 µg/ml	1×10^6	1
o	1 ml	-	tet - 2.5	100	2.5 µg/ml	1×10^7	3.3
p	-	1 ml	tet - 2.5	100	2.5 µg/ml	3.3×10^6	-
q	1 ml	-	tet - 5	100	5 µg/ml	$>1 \times 10^8$	-
r	-	1 ml	tet - 5	100	5 µg/ml	$>1 \times 10^8$	-
s	1 ml	-	tet - 10	100	10 µg/ml	$>1 \times 10^8$	-
t	-	1 ml	tet - 10	100	10 µg/ml	$>1 \times 10^8$	-

One ml of murine macrophages, J774 (5×10^5/ml) was combined with YGP, YGP: alginate – tet or tetracycline of various concentration as summarized in Table 6, above.

The J774 cells were cultured overnight in medium (DMEM containing 10% fetal calf serum without antibiotics or glutamine). The cultures were incubated with medium alone, tetracycline diluted in medium or particles diluted in medium for 1 hour with rotation at 37 degrees Celsius to permit phagocytosis of the particles. The microbial killing assay was set up in 96 well plates. The cultures were diluted in medium and incubated overnight to allow for metabolism and release of tet from phagocytosed YGP: alginate – tet particles. Bacterial challenge was added as indicated in Table 6 and the cultures were incubated 2 hours at 37 degrees Celsius in a CO_2 incubator to permit *S. aureus* phagocytosis and killing by the J774 murine macrophages. After this incubation, 200 µl LB Broth (Luria-Bertani Broth: 1.0% tryptone, 0.5% yeast extract, 1.0% NaCl) was added to each culture to lyze the macrophages. Cultures were incubated at 37 degrees Celsius in an incubator to permit outgrowth of surviving *S. aureus*. Growth was monitored by change in pH as indicated by phenol red. The effects of

YGP, YGP: alginate – tet or tetracycline were compared. The results are provided in the two right-most columns of Table 6.

About 7.5 x 10^6 YGP: alginate – tet particles produced an effect on macrophages roughly equivalent to about 2.5 μg/ml tetracycline HCl. The macrophages alone were relatively less effective than macrophages treated with tetracycline in either mode, and about as effective as macrophages treated with empty YGP alone. Macrophages in combination with free tetracycline in solution were not much more effective than tetracycline alone. Macrophages treated with YGP: alginate – tet particles showed significant synergy. In general, the results demonstrate that phagosome delivery of tetracycline into J774 macrophage cells enhances the killing capacity of J774 macrophage cells for *S. aureus*.

Example 15: Loading of Protein into YGP

The utility of the delivery system of the present invention for the retention, transport and delivery of therapeutic peptides or proteins, vaccine antigens or other peptides or proteins was evaluated using the mixed proteins of fetal calf serum. Yeast cell wall particles used were YGP, YGP-PEI and YGP-chitosan prepared as described above. Stock solutions were 45 ng/μl fetal calf serum (FCS) (Fetal Bovine Serum, JRH Biosciences, Lenexa, KS), 0.2% PEI (Sigma Chemical Co., St. Louis, MO) in TE, 0.05 M phosphate buffer, pH 7.2 (P buffer) and 0.05 M phosphate buffer, pH 7.2, 1 M NaCl (P + salt buffer).

Four μl of FCS were added to 1 mg of YGP, YGP-P or YGP-CN in microcentrifuge tubes as indicated in Table 7 and the resulting mixture was incubated 60 minutes at room temperature to allow the liquid to be absorbed by the particles. After the incubation, 200 μl phosphate buffer or 200 μl PEI was as indicated in Table 7 and the resulting mixture was incubated 60 minutes at room temperature. After the incubation, 0.5 ml phosphate buffer was added, and after a further 5 minute incubation, the tubes were sonicated to produce single particles. The particles were pelleted by centrifuging at 10, 000 rpm for 10 minutes and the supernatants were removed to fresh tubes. 0.5 ml 0.05M sodium phosphate buffer, pH 7.2 + 1M NaCl was added to the pellets, and after a further 5 minute incubation, the tubes were centrifuged at 10, 000 rpm for 10 minutes and the high salt elution supernatants were removed to fresh tubes. The protein content of the supernatants was measured by absorbance at 280 nm.

Table 7					
Tube	YGP	1° Load	2 ° Load	P buffer (µl)	P+Salt buffer (µl)
1	-	4 µl FCS	200 µl P buffer	500	500
2	YGP	4 µl FCS	200 µl P buffer	500	500
3	YGP	4 µl FCS	200 µl 2% PEI	500	500
4	YGP-PEI	4 µl FCS	200 µl P buffer	500	500
5	YGP-CN	4 µl FCS	200 µl P buffer	500	500

The protein loading results are shown in Table 8. YGP particles without a trapping molecule trapped only 5% of the presented protein. YGP particles that were loaded first with FCS protein and then exposed to PEI retained 47% of the protein load. YGP particles that were preloaded with a trapping polymer such as PEI or chitosan before exposure to the protein load such retained 68% and 60%, respectively, of the protein load.

Table 8							
Tube	YGP	Payload	Trapping Polymer	Unbound Protein (ng)	% Unbound Protein	Bound Protein (ng)	% Bound Protein
1	-	FCS	P buffer	180	100	-	-
2	YGP	FCS	P buffer	180	95	10	5
3	YGP	FCS	2% PEI	120	63	70	47
4	YGP-PEI	FCS	P buffer	60	32	130	68
5	YGP-CN	FCS	P buffer	80	40	120	60

The results demonstrate that serum proteins are not effectively loaded and trapped into YGP without trapping polymers. Using YGP that were preloaded with trapping polymers before exposure to the payload proteins resulted in increased protein trapping. Alternatively, proteins can be trapped inside YGP by first loading the protein, and then adding a soluble trapping polymer to sequester the protein within the particle.

Example 16: Comparison of Various Methods of Loading DNA into YGP

Several methods of loading salmon sperm DNA into YGP, YGP containing low molecular weight (LMW) chitosans or YGP containing high molecular weight (HMW) chitosans were evaluated.

a. Capillary loading followed by ethanol precipitation

Salmon sperm DNA Sigma, St. Louis, MO) was sheared by 40 passes through 18 gauge needle and diluted to a concentration of 0.1 mg/ml in 50 mM TE (Tris-HCl, pH 8, 2 mM EDTA). Loading volumes of the DNA solution were determined and mixed in centrifuge tubes in duplicate with 100 mg aliquots of YGP, YGP: LMW chitosan or YGP: HMW chitosan as in Example 2 and incubated 1 hour. The incubated mixtures were ethanol precipitated by adding 1.5 ml ethanol to each tube. The insoluble products were collected by centrifugation at 2,000 rpm for 10 minutes. 10ml TE was added to each tube, incubated for 1 hr at 37 degrees Celsius,centrifuged 2,000 rpm for 10 minutes to sediment the insoluble YGP and the DNA content of the supernatant was determined by absorbance at 260 nm. The amount of DNA remaining in the YGP was calculated.

b. DNA loading by absorption

Loading volumes of the DNA solution were mixed in centrifuge tubes in duplicate with 100 mg aliquots of YGP, YGP: LMW chitosan or YGP: HMW chitosan as in Example 4a and incubated 1 hour. 10ml TE was added to each tube, incubated for 1 hr at 37 degrees Celsius, centrifuged 2,000 rpm for 10 minutes to sediment the insoluble YGP. The DNA content of the supernatant was determined by absorbance at 260 nm. The amount of DNA remaining in the YGP was calculated.

c. DNA loading by CTAB trapping

Loading volumes of the DNA solution were mixed in centrifuge tubes in duplicate with 100 mg aliquots of YGP, YGP: LMW chitosan or YGP: HMW chitosan as in Example 4 and incubated 1 hour. The incubated mixtures were precipitated by adding 1.5 ml 2% hexadecyltrimethylammoniumbromide (also known as cetyltrimethylammonium bromide or CTAB) solution to each tube. 10ml TE was added to each tube, which was incubated for 1 hr at 37 degrees Celsius, and centrifuged 2,000 rpm for 10 minutes to sediment the insoluble YGP. The DNA content of the supernatant was determined by absorbance at 260 nm. The amount of DNA remaining in the YGP was calculated.

The amount of DNA remaining in the YGP was calculated.

The results are presented in Table 9, below.

Table 9			
	% DNA bound in YGP		
Method	YGP	YGP:LMW chitosan	YGP: HMW chitosan
Direct Loading	<1%	32%	70%
Direct Loading + Ethanol	<1%	Not done	Not done
Direct Loading CTAB trapping	>99%	>99%	99%
Absorption Loading	<1%	5%	12%

Simple DNA loading or precipitation failed to effectively load and trap DNA into the YGP. In contrast, the use of the cationic trapping polymer, chitosan, resulted in the formation of chitosan-DNA complexes that can trap DNA inside YGP. In addition, the cationic agent CTAB can be effectively used to trap loaded DNA into YGP.

Example 17: DNA Loading and Trapping

Fluorescent salmon sperm DNA was prepared by mixing 1 ml of a 1 mg/ml solution of salmon sperm DNA in 0.1M carbonate buffer pH 9.2 with 100 µl of a 1 mg/ml suspension of DTAF in 10 mM carbonate buffer ph 9.2. After overnight incubation at 37 degrees Celsius, 200 µl 1M Tris-HCl pH 8.3 was added and incubated for 15 minutes at room temperature. Then, 100 µl 1M NaCl and 3 mls ethanol were added to ethanol precipitate the DNA. After storage at -20C overnight, the ethanol precipitate was collected by centrifugation at 10,000 rpm 15 minutes. The ethanol precipitate was washed with 70% ethanol until supernatant was clear and resuspended in 1 ml TE.

The YGP suspensions were incubated for 30 minutes at room temperature. After the incubation, 0.45 ml 95% ethanol was added to one set (YGP, YGP-P, YGP-Chitosan) of three tubes, 0.2 ml 2% PEI was added to two sets of three tubes and 0.2 ml 2% CTAB was added to another set of three tubes. After 30 minutes incubation at room temperature, 0.2 ml 2% CTAB was added to one set of the PEI tubes and incubation proceeded for a further 30 minutes. Ethanol (1ml, 95%) was added and the YGPs were stored overnight at –20 degrees Celsius. The YGP suspensions were washed with 70% ethanol and resuspended in 0.5 ml PBS. Results were evaluated by fluorescence microscopy, and are shown in Table 10.

Table 10

Particle	Treatment	YGP pellet	Fluorescence Microscopy Observation
YGP	ethanol	White	Not fluorescent
YGP-CN	ethanol	Yellow	Internal particle fluorescence
YGP-P	ethanol	Yellow	Internal particle fluorescence
YGP	2% PEI	Yellow	Internal particle fluorescence
YGP-CN	2% PEI	Yellow	Weak internal particle fluorescence
YGP-P	2% PEI	Yellow	Weak internal particle fluorescence
YGP	2% CTAB	Yellow	Internal particle fluorescence
YGP-CN	2% CTAB	Yellow	Strong internal particle fluorescence
YGP-P	2% CTAB	Yellow	Strong internal particle fluorescence
YGP	2% PEI/2% CTAB	Yellow	Strong internal particle fluorescence
YGP-CN	2% PEI/2% CTAB	Yellow	Internal particle fluorescence
YGP-P	2% PEI/2% CTAB	Yellow	Internal particle fluorescence

No significant trapping of fluorescent-labeled DNA occurred if only simple ethanol precipitation without a trapping polymer was used, demonstrating that the prior art technology is not effective as a DNA delivery system. Fluorescent-labeled DNA was clearly being trapped by cationic trapping polymers PEI or chitosan, or with the cationic detergent CTAP inside YGP particles. The best DNA trapping occurred when a combination of trapping polymer and CTAB was used, such as YGP:PEI: DNA:CTAB, YGP:chitosan:DNA: CTAB or YGP:DNA:PEI:CTAB.

Example 18: Fluorescently Labeled Plasmid DNA Loading and Trapping

YGP containing pIRES plasmid was prepared for transfection and expression of encoded EGFP in J774 cells, a murine macrophage derived cell line. Cationic trapping agents used included cationic polymers such as polyethylenimine (PEI), CytoPure™, a proprietary, commercially available, water-soluble cationic polymer transfection reagent (Qbiogene, Inc., CA), chitosan and a cationic detergent hexadecyltrimethyl-ammoniumbromide (CTAB). A preferred PEI is JetPEI, a commercially available linear polyethylenimine cationic polymer transfection reagent (Qbiogene, Inc., CA).

pIRES-EGFP (Clonetech, CA) contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) between the MCS and the EGFP (enhanced green fluorescent protein) coding region. This permits both the gene of interest (cloned into the MCS) and the EGFP gene to be translated from a single bicistronic mRNA. pIRES-EGFP is designed for the efficient selection (by flow cytometry or other methods) of transiently transfected mammalian cells expressing EGFP and another protein of interest. To optimize the selection of

cells expressing high levels of the protein of interest, pIRES-EGFP utilizes a partially disabled IRES sequence (1). This attenuated IRES leads to a reduced rate of translation initiation at the EGFP start codon relative to that of the cloned gene. This enables the selection of those cells in which the mRNA, and hence the target protein, is produced at high levels to compensate for a suboptimal rate of translation of EGFP. This vector can also be used to express EGFP alone or to obtain stably transfected cell lines without time-consuming drug and clonal selection. EGFP is a red-shifted variant of wild-type GFP that has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maximum = 488 nm; emission maximum = 509 nm) EGFP encodes the GFPmut1 variant, which contains the amino acid substitutions Phe-64 to Leu and Ser-65 to Thr. These mutations increase the brightness and solubility of GFP, primarily due to improved protein folding properties and efficiency of chromophore formation. EGFP also contains an open reading frame composed almost entirely of preferred human codons. This leads to more efficient translation and, hence, higher expression levels in eukaryotic cells, relative to wild type GFP.

Solutions prepared were: pIRES EGFP plasmid DNA, 0.72 μg/μl in water, 0.2% w/v PEI (Sigma) in TE, 2 μl CytoPure (Qbiogene) + 48 μl 0.15M NaCl, 2 μl JetPEI (Qbiogene) + 48 μl TE, 0.2% Spermidine in TE, 2% (aq) CTAB and phosphate buffered saline (PBS).

Fluorescent pIRES plasmid DNA was prepared by mixing 1 ml of a 1 mg/ml solution of pIRES DNA in 0.1M carbonate buffer pH 9.2 with 100 μl of a 1 mg/ml suspension of DTAF in 10 mM carbonate buffer pH 9.2. After overnight incubation at 37 degrees Celsius, 200 μl 1M Tris-HCl pH 8.3 was added and incubated for 15 minutes at room temperature. Then 100 μl 1M NaCl and 3 ml ethanol were added to ethanol precipitate the DNA. After storage at -20 degrees Celsius overnight, the ethanol precipitate was collected by centrifugation at 10,000 rpm 15 minutes. The ethanol precipitate was washed with 70% ethanol until supernatant was clear and resuspended in 1 ml TE.

The YGP suspensions were incubated for 30 minutes at room temperature. After the incubation, 0.45 ml 95% ethanol was added to one set (YGP, YGP-P, YGP-Chitosan) of three tubes, 0.2 ml 2% PEI was added to two sets of three tubes and 0.2 ml 2% CTAB was added to another set of three tubes. After 30 minutes incubation at room temperature, 0.2 ml 2% CTAB was added to one set of the PEI tubes and incubation proceeded for a further 30 minutes. Ethanol (1ml, 95%) was added and the YGPs were stored overnight at –20 degrees Celsius. The YGP suspensions were washed with 70% ethanol and resuspended in 0.5 ml PBS.

J774 murine macrophages were plated in six well plates at a density of 2.5 x10^5 cells per well and incubated overnight as described in Example 14. The transfections were performed as summarized in Table 11. The particles were added to the culture medium at a 10 particle per cell ratio and the plates were swirled to distribute particles. The cells were incubated for 4 hours. At end of the incubation period, the culture medium was removed, the cells were washed with PBS and fixed in 0.4% formalin in PBS.

Table 11

Tube	pIRES µg/µl	vol µl	YGP mg	0.2% PEI in TE	0.2% Chitosan in Acetate buffer pH 5.5	2% CTAB	Ethanol
1	-	-	1	200 µl	-	200 µl	800 µl
2	-	-	1	-	200 µl	200 µl	800 µl
3	1.8	4	1	200 µl	-	200 µl	800 µl
4	1.8	4	1	-	200 µl	200 µl	800 µl

Fluorescent DNA-containing particles and J774 cells incubated with fluorescent DNA-containing particles were evaluated by fluorescence microscopy, and results are summarized in Table 12 and shown in Figures 3A and 3B.

Table 12

Particle Type	Treatment	Color of Pellet	Microscopic Examination of Particles
YGP	ethanol	White	No fluorescence
YGP-CN	ethanol	Yellow	Intracelluar fluorescent particles
YGP-P	ethanol	Yellow	Intracelluar fluorescent particles
YGP	2% PEI	Yellow	Intracelluar fluorescent particles
YGP-CN	2% PEI	Yellow	Intracelluar fluorescent particles
YGP-P	2% PEI	Yellow	Intracelluar fluorescent particles
YGP	2% CTAB	Yellow	Intracelluar fluorescent particles
YGP-CN	2% CTAB	Yellow	Intracelluar fluorescent particles
YGP-P	2% CTAB	Yellow	Intracelluar fluorescent particles
YGP	2% PEI/2% CTAB	Yellow	Figures 3A & 3B; strongly fluorescent Intracellular particles
YGP-CN	2% PEI/2% CTAB	Yellow	Intracelluar fluorescent particles
YGP-P	2% PEI/2% CTAB	Yellow	Intracelluar fluorescent particles

Figure 3A is a reversed contrast (negative) grayscale image of a color light photomicrograph of cells exposed to YGP particles loaded with fluorescent labeled pIRES plasmid with PEI as the cationic trapping polymer and CTAB as a cationic detergent, indicating a cell 310. Figure 3B is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph of the same field of cells showing bright staining representing fluorescent YGP particles containing fluorescent plasmid DNA internalized by the same cell 310 indicated in Figure 3B.

Example 19: EGFP Expression By J774 Murine Macrophages Incubated With YGP:pIRES

The pIRES plasmid DNA was not fluorescently labeled in this Example, rather the functional expression of the green fluorescent protein (GFP) encoded by pIRES was used as a demonstration of uptake of loaded yeast cell wall particles, intracellular release of the pIRES DNA and expression of the GFP as evidenced by the production of fluorescence.

The YGP: pIRES formulations were prepared as summarized in Table 12, below. DNA was prepared from dilutions in deionized water of 1 mg/ml stock. The indicated amount of DNA solution was added to YGP and incubated for at least 30 minutes to allow for liquid absorption. The indicated amount of 0.2% PEI in TE or 0.2% chitosan in acetate buffer was added and the mixture was allowed to incubate for 5 minutes before sonication to produce single particles. After a further incubation of at least 30 minutes, the indicated amount of 2% CTAB was added. After an additional 5 minute incubation, the tubes were vortex mixed and incubated again for at least 30 minutes. The indicated amount of 95% ethanol was added. Each tube was then mixed and stored at –20 Celsius overnight. The YGP:pIRES formulated particles were then centrifuged, washed twice in 70% ethanol, collected by centrifugation at 10,000 rpm for 5 minutes, resuspended in 0.5 ml sterile PBS and sonicated to produce single particles. The number of particles per ml was counted and each formulation was and stored at –20 degrees Celsius.

J774 murine macrophages were plated in 6 well plates at a density of 2.5 $x10^5$ cells per well and incubated overnight as described in Example 14. The transfections were performed as summarized in Table 11, above. The particles were added to the culture medium at a 10 particle per cell ratio and the plates were swirled to distribute particles. The cells were fed daily and incubated for 2 days. At end of the incubation period, the culture medium was removed the cells were washed with PBS and fixed in 0.4% formalin in PBS.

The results are summarized in Table 13 and shown in Figures 4A – C. Cells were examined using fluorescence microscopy. Eighty nine percent of J774 cells took up YGP-F particles (Table 13, well 1B, Figure 4A). EGFP expression was evident in >80% of J774 cells as punctate fluorescence in vacuoles in wells 1E (Figure 4B) and 1F (Figure 4C).

Table 13				
Well	Description	YGP/Cell	volume	Appearance
1A	No Treatment Control	0	-	No detectible GFP fluorescent particles
1B	YGPF Particle Uptake Control	10	10 µl 1/10	Figure 4A, showing phagocytosis of fluorescent YGFP particles
1C	YGP empty PEI/CTAB Control	10	11 µl 1/10	No detectible GFP fluorescent particles
1D	YGP empty Chitosan/CTAB Control	10	5 µl 1/10	No detectible GFP fluorescent particles
1E	YGP pIRES PEI/CTAB	10	10 µl 1/10	Figure 4B, showing fluorescent GFP expression in cells
1F	YGP pIRES Chitosan/CTAB	10	6.5 µl 1/10	Figure 4C, showing fluorescent GFP expression in cells

Figure 4A is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph of cells, e.g., an indicated cell 410, exposed to fluorescent labeled YGP particles, Figure 4B is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph of cells, e.g., an indicated cell 420, expressing GFP from pIRES DNA delivered by YGP with a cationic trapping polymer polyethylenimine (PEI) and cationic detergent hexadecyltrimethylammoniumbromide (also known as cetyltrimethylammonium bromide or CTAB) and Figure 4C is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph of cells, e.g., an indicated cell 430, expressing GFP from pIRES DNA delivered by YGP with a cationic trapping polymer chitosan and cationic detergent CTAB.

Example 20: Fluorescent DNA, Oligonucleotide and siRNA Oligonucleotide Delivery into J774 Cells Using YGP-Cation Trapping Polymer Technology

The following materials were used: YGP:Fluorescent salmon sperm DNA:PEI:CTAB particles, YGP:Fluorescent oligonucleotide:PEI:CTAB particles, and YGP:Fluorescent siRNA:PEI:CTAB. The fluorescent oligonucleotide was an 18 mer synthesized by Sigma Genosys with a fluorescein residue attached to the 5' end:

5' Fluorescein-TTGGTCATCCATGGCTCT 3' SEQ ID NO:1.

The fluorescent siRNA was a 21 mer non-silencing control siRNA synthesized with a fluorescein residue attached to the 5' end (Qiagen, Valencia, CA, Catalog No. 1022079):

5' Fluorescein-UUCUCCGAACGUGUCACGUdTdT 3' SEQ ID NO:2.

J774 murine macrophages were plated in 6 well plates at a density of 2.5 x10^5 cells per well and incubated overnight as described in Example 14. The transfections were performed as summarized in Table 14. The control and polynucleotide-loaded particles were added to the culture medium and the plates were swirled to distribute particles. The cells were fed daily and incubated for 24 hours. At end of the incubation period, the culture medium was removed the cells were washed with PBS and fixed in 0.4% formalin in PBS.

Table 14

Well	Cells	YGP/Cell Ratio	Particles
1A	J774	0	-
1B	J774	10	YGPF
1C	J774	10	YGP DNAF
1D	J774	10	YGP oligoF
1E	J774	10	YGP RNAiF

The results are illustrated in Figures 5A – I. Cells were examined using fluorescence microscopy and FACS. 92% of J774 cells took up YGP-F particles (Table 14, well 1B, Figure 5A). Fluorescent oligonucleotide (SEQ ID NO:1) delivery was evident in >80% of J774 cells as punctate endosomal fluoresence and diffuse cytoplasmic fluorescence. Fluorescent non-silencing siRNA (SEQ ID NO:1) delivery was evident in >80% of J774 cells as punctate endosomal fluorescence and diffuse cytoplasmic fluorescence.

Figure 5A is a reversed contrast (negative) grayscale image of a color combined light and fluorescence photomicrograph of cells, e.g., an indicated cell 510, exposed to fluorescent labeled YGP particles; Figure 5B is a graphic representation of the results of a fluorescence activated cell sorting (FACS) study showing a major peak 520 representing the distribution of signals from cells that have internalized fluorescent labeled YGP particles and a minor peak 530 representing the distribution of signals from cells without fluorescent labeled YGP particles; Figure 5C is a reversed contrast (negative) grayscale image of a color light photomicrograph of cells, e.g., an indicated cell 540, exposed to YGP particles containing fluorescent labeled DNA, a cationic trapping polymer PEI and cationic detergent CTAB; Figure 5D is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph of the same field of cells showing the same indicated cell 540, Figure 5E is a graphic representation of the results of a FACS study showing a major peak 610 representing the distribution of signals from cells that have internalized YGP particles with fluorescent DNA payload and a shoulder 620 representing the

distribution of signals from cells without YGP particles; Figure 5F is a reversed contrast (negative) grayscale image of a color light photomicrograph of cells, e.g., an indicated cell 710, incubated with YGP particles containing fluorescent labeled antisense RNA, PEI and CTAB; Figure 5G is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph of the same field of cells showing the same indicated cell 710 containing internalized YGP particles with fluorescent antisense RNA payload; Figure 5H is a reversed contrast (negative) grayscale image of a color light micrograph of cells, e.g., an indicated cell 810, incubated with YGP particles containing fluorescent labeled siRNA, PEI and CTAB and Figure 5I is a reversed contrast (negative) grayscale image of a color fluorescence photomicrograph of the same field of cells showing the same indicated cell 810 containing internalized YGP particles with fluorescent RNAi payload.

In summary, fluorescent DNA, oligonucleotide or siRNA payloads loaded into YGP using a cationic trapping polymer efficiently delivers the payload into J774 cells. Payloads are released from the endosomal compartment within 24 hours into the cytoplasm and nuclear compartments.

The claims should not be read as limited to the described order or elements unless stated to that effect. Therefore, all embodiments that come within the scope and spirit of the following claims and equivalents thereto are claimed as the invention.

CLAIMS

What is claimed:

1. A particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan and a payload trapping molecule.

2. The particulate delivery system of claim 1 further comprising a payload molecule, wherein the payload molecule and the payload trapping molecule are soluble in the same solvent system.

3. The particulate delivery system of claim 2 wherein the solvent system comprises water.

4. The particulate delivery system of claim 1 wherein the extracted yeast cell wall further comprises less than 90 weight percent beta-glucan.

5. The particulate delivery system of claim 1 wherein the extracted yeast cell wall further comprises more than 50 weight percent chitin.

6. The particulate delivery system of claim 1 wherein the extracted yeast cell wall further comprises more than 30 weight percent mannan.

7. The particulate delivery system of claim 1 wherein the extracted yeast cell wall further comprises more than 1 weight percent protein.

8. The particulate delivery system of claim 1 wherein the payload trapping molecule is a polysaccharide selected from the group consisting of agarose, an alginate, a xanthan, a dextran, a chitosan, a galactomannan gum, a derivative thereof and a mixture thereof.

9. The particulate delivery system of claim 1 wherein the payload trapping molecule is polyacrylamide.

10. The particulate delivery system of claim 1 wherein the payload trapping molecule is a polyamide.

11. The particulate delivery system of claim 1 wherein the payload trapping molecule is selected from the group consisting of a cationic polymer, an anionic polymer, a cationic detergent, an anionic detergent and a mixture thereof.

12. The payload trapping molecule of claim 11 wherein the cationic polymer is selected from the group consisting of chitosan, polyethylenimine and poly-L-lysine.

13. The payload trapping molecule of claim 11 wherein the payload trapping molecule is a mixture of a cationic polymer and a cationic detergent.

14. The payload trapping molecule of claim 11 wherein the cationic polymer is selected from the group consisting of a protein, a polypeptides, a short synthetic peptide, a helical amphiphilic peptide, a cationic dendrimers, glucaramide polymer, a N-substituted glycine oligomer, poly(2-methyl-acrylic acid 2-[(2-dimethylamino)-ethyl)-methyl-amino] -ethyl ester), poly(2-dimethylamino ethyl)- methacrylate and mixtures thereof.

15. The payload trapping molecule of claim 11 wherein the anionic polymer is selected from the group consisting of alginate and xanthan.

16. The payload trapping molecule of claim 11 wherein the cationic detergent is hexadecyltrimethylammoniumbromide.

17. The particulate delivery system of claim 1 wherein the payload trapping molecule is selected from the group consisting of a cationic polyelectrolyte, an anionic polyelectrolyte and an amphoteric polyelectrolyte.

18. The particulate delivery system of claim 17 wherein the cationic polyelectrolyte is selected from the group consisting of a copolymer of vinyl pyrollidone and quaternary methyl methacrylate, a substituted polyacrylamide, polyethyleneimine, polypropyleneimine, a polyamine homopolymer, a polyamine co-polymer, polydiallyl

dimethyl ammonium chloride, substituted dextrans; modified guar gum, a substituted protein, a polyamino acid, spermine and spermidine.

19. The particulate delivery system of claim 17 wherein the anionic polyelectrolyte is selected from the group consisting of a copolymer of methyl vinyl ether and maleic anhydride, a copolymer of methyl vinyl ether and maleic acid, alginic acid a carboxymethyl cellulose, a substituted polyacrylamide, a polyacrylic acid, a polystyrene sulfonic acid, a dextran sulphates, a substituted saccharide, heparin and pharmaceutically acceptable salts.

20. The particulate delivery system of claim 2 wherein the payload molecule is selected from the group consisting of a polynucleotide, a peptide, a protein, a small organic active agent, a small inorganic active agent and a mixture thereof.

21. The particulate delivery system of claim 20 wherein the polynucleotide is selected from the group consisting of an oligonucleotide, an antisense construct, a siRNA, an enzymatic RNA, a recombinant DNA construct and a mixture thereof.

22. The particulate delivery system of claim 21 wherein the recombinant DNA construct is an expression vector comprising a control element operatively linked to an open reading frame encoding a protein.

23. The particulate delivery system of claim 22 wherein the protein encoded by the open reading frame is a structural protein, a protein having enzymatic activity, a membrane protein, a DNA binding protein or a signaling protein.

24. The particulate delivery system of claim 22 wherein the protein encoded by the open reading frame is an antigenic protein.

25. The particulate delivery system of claim 19 wherein the polynucleotide comprises a nucleotide sequence that restores the function of an absent, defective or inhibited gene.

26. The particulate delivery system of claim 22 wherein the protein encoded by the open reading frame is a protein that produces a therapeutic effect in an individual having a genetic disorder.

27. The particulate delivery system of claim 25 wherein the genetic disorder is Aarskog-Scott syndrome, Aase syndrome, achondroplasia, acrodysostosis, addiction, adreno-leukodystrophy, albinism, ablepharon-macrostomia syndrome, alagille syndrome, alkaptonuria, alpha-1 antitrypsin deficiency, Alport's syndrome, Alzheimer disease, asthma, autoimmune polyglandular syndrome, androgen insensitivity syndrome, Angelman syndrome, ataxia, ataxia telangiectasia, atherosclerosis, attention deficit hyperactivity disorder (ADHD), autism, baldness, Batten disease, Beckwith-Wiedemann syndrome, Best disease, bipolar disorder, brachydactyly, breast cancer, Burkitt lymphoma, chronic myeloid leukemia, Charcot-Marie-Tooth disease, Crohn's disease, cleft lip, Cockayne syndrome, Coffin Lowry syndrome, colon cancer, congenital adrenal hyperplasia, Cornelia de Lange syndrome, Costello syndrome, Cowden syndrome, craniofrontonasal dysplasia, Crigler-Najjar syndrome, Creutzfeldt-Jakob disease, cystic fibrosis, deafness, depression, diabetes, diastrophic dysplasia, DiGeorge syndrome, Down's syndrome, dyslexia, Duchenne muscular dystrophy, Dubowitz syndrome, ectodermal dysplasia, Ellis-van Creveld syndrome, Ehlers-Danlos, epidermolysis bullosa, epilepsy, essential tremor, familial hypercholesterolemia, familial Mediterranean fever, fragile X syndrome, Friedreich's ataxia, Gaucher disease, glaucoma, glucose galactose malabsorption, glutaricaciduria, gyrate atrophy, Goldberg Shprintzen syndrome (velocardiofacial syndrome), Gorlin syndrome, Hailey-Hailey disease, hemihypertrophy, hemochromatosis, hemophilia, hereditary motor and sensory neuropathy (HMSN), hereditary non polyposis colorectal cancer (HNPCC), Huntington's disease, immunodeficiency with hyper-IgM, juvenile onset diabetes, Klinefelter's syndrome, Kabuki syndrome, Leigh's disease, long QT syndrome, lung cancer, malignant melanoma, manic depression, Marfan syndrome, Menkes syndrome, miscarriage, mucopolysaccharide disease, multiple endocrine neoplasia, multiple sclerosis, muscular dystrophy, myotrophic lateral sclerosis, myotonic dystrophy, neurofibromatosis, Niemann-Pick disease, Noonan syndrome, obesity, ovarian cancer, p53 tumor suppressor, pancreatic cancer, Parkinson disease, paroxysmal nocturnal hemoglobinuria, Pendred

syndrome, peroneal muscular atrophy, phenylketonuria (PKU), polycystic kidney disease, Prader-Willi syndrome, primary biliary cirrhosis, prostate cancer, REAR syndrome, Refsum disease, retinitis pigmentosa, retinoblastoma, Rett syndrome, Sanfilippo syndrome, schizophrenia, severe combined immunodeficiency, sickle cell anemia, spina bifida, spinal muscular atrophy, spinocerebellar atrophy, SRY: sex determination, sudden adult death syndrome, Tangier disease, Tay-Sachs disease, thrombocytopenia absent radius syndrome, Townes-Brocks syndrome, tuberous sclerosis, Turner syndrome, Usher syndrome, von Hippel-Lindau syndrome, Waardenburg syndrome, Weaver syndrome, Werner syndrome, Williams syndrome, Wilson's disease, xeroderma pigmentosum or Zellweger syndrome.

28. The particulate delivery system of claim 20 wherein the protein is selected from the group consisting of growth hormone, prolactin, placental lactogen, erythropoietin, thrombopoietin, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-9, interleukin-10, interleukin-11, interleukin-12 (p35 subunit), interleukin-13, interleukin-15, oncostatin M, ciliary neurotrophic factor, leukemia inhibitory factor, alpha interferon, beta interferon, gamma interferon, omega interferon, tau interferon, granulocyte-colony stimulating factor, granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor, cardiotrophin-1growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; factor VIIIC, factor IX tissue factor, von Willebrands factor, Protein C, atrial natriuretic factor, lung surfactant, urokinase, tissue-type plasminogen activator, bombazine, thrombin, alpha tumor necrosis factor, beta tumor necrosis factor, enkephalinase; RANTES, human macrophage inflammatory protein, serum albumin, mullerian-inhibiting substance, relaxin A-chain, relaxin B-chain, prorelaxin, mouse gonadotropin-associated peptide, DNase, inhibin, activin, vascular endothelial growth factor, a hormone receptor, a growth factors receptor, an integrin, protein A, protein D, a rheumatoid factor, a neurotrophic factor, bone-derived neurotrophic factor (BDNF), neurotrophin-3, neurotrophin-4, neurotrophin-5, or neurotrophin-6, NGF-beta, platelet-derived growth factor (PDGF); alpha fibroblast growth factor, beta alpha fibroblast growth factor, epidermal growth factor, transforming

growth factor-alpha, transforming growth factor-beta1, transforming growth factor-beta2, transforming growth factor-beta3, transforming growth factor-beta4, transforming growth factor-beta5, insulin-like growth factor-I, insulin-like growth factor-II, des(1-3)-insulin-like growth factor-I, a insulin-like growth factor binding protein, CD3, CD4, CD8, CD19, CD20, an osteoinductive factor, an immunotoxin, a bone morphogenetic protein, a T-cell receptor, surface membrane proteins, decay accelerating factor, a viral antigen, a transport protein, homing receptor, an addressin, a regulatory protein, an immunoadhesin, an antibody and biologically active fragments or variants thereof.

29. The particulate delivery system of claim 22 wherein the protein encoded by the open reading frame is selected from the group consisting of growth hormone, prolactin, placental lactogen, erythropoietin, thrombopoietin, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-9, interleukin-10, interleukin-11, interleukin-12 (p35 subunit), interleukin-13, interleukin-15, oncostatin M, ciliary neurotrophic factor, leukemia inhibitory factor, alpha interferon, beta interferon, gamma interferon, omega interferon, tau interferon, granulocyte-colony stimulating factor, granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor, cardiotrophin-1growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; factor VIIIC, factor IX tissue factor, von Willebrands factor, Protein C, atrial natriuretic factor, lung surfactant, urokinase, tissue-type plasminogen activator, bombazine, thrombin, alpha tumor necrosis factor, beta tumor necrosis factor, enkephalinase; RANTES, human macrophage inflammatory protein, serum albumin, mullerian-inhibiting substance, relaxin A-chain, relaxin B-chain, prorelaxin, mouse gonadotropin-associated peptide, DNase, inhibin, activin, vascular endothelial growth factor, a hormone receptor, a growth factors receptor, an integrin, protein A, protein D, a rheumatoid factor, a neurotrophic factor, bone-derived neurotrophic factor (BDNF), neurotrophin-3, neurotrophin-4, neurotrophin-5, or neurotrophin-6, NGF-beta, platelet-derived growth factor (PDGF); alpha fibroblast growth factor, beta alpha fibroblast growth factor, epidermal growth factor, transforming growth factor-alpha, transforming growth factor-beta1, transforming growth factor-beta2, transforming growth factor-beta3,

transforming growth factor-beta4, transforming growth factor-beta5, insulin-like growth factor-I, insulin-like growth factor-II, des(1-3)-insulin-like growth factor-I, a insulin-like growth factor binding protein, CD3, CD4, CD8, CD19, CD20, an osteoinductive factor, an immunotoxin, a bone morphogenetic protein, a T-cell receptor, surface membrane proteins, decay accelerating factor, a viral antigen, a transport protein, homing receptor, an addressin, a regulatory protein, an immunoadhesin, an antibody and biologically active fragments or variants thereof.

30. The particulate delivery system of claim 20 wherein the small organic active agent is an oligomer of heterocyclic polyamides that binds to the minor groove of double stranded DNA in a sequence specific manner.

31. The particulate delivery system of claim 20 wherein the small organic active agent is an oligomer having monomeric subunits selected from the group consisting of N-methylimidazole carboxamide, N-methylpyrrole carboxamide, beta-alanine and dimethylaminopripylamide.

32. The particulate delivery system of claim 19 wherein the small organic active agent is a contraceptive agent, a gastrointestinal therapeutic agent, a non-steroidal antifertility agent, a parasympathomimetic agent, a psychotherapeutic agent, a major tranquilizer, a minor tranquilizer, a rhinological decongestant, a sedative-hypnotic, a steroid, a sulfonamide, a vaccine; a vitamin, a nutrient, an antimalarial, an anti-migraine agent, an anti-Parkinson agent, an anti-spasmodic, an anticholinergic agent, an antitussive, a bronchodilator, a cardiovascular agent; an anti-hypertensive agent, a coronary vasodilator, an organic nitrate, an alkaloid, an analgesic, a narcotic, an anti-cancer agent, an anti-convulsant, an anti-emetic, an anti-inflammatory agent, a cytotoxic drug or an antibiotic.

33. The particulate delivery system of claim 20 wherein the small organic active agent is an antibiotic selected from the group consisting of a cephalosporin, chloramphenical, gentamicin, kanamycin A, kanamycin B, a penicillin, ampicillin, streptomycin A,

antimycin A, chloropamtheniol, metronidazole, oxytetracycline, penicillin G, a tetracycline and mixtures thereof.

34. An article of manufacture comprising a first container containing a payload molecule selected from the group consisting of a nucleic acid composition, protein composition, small organic molecule and mixtures thereof, a second container containing a particulate delivery system comprising a yeast cell wall particle and a payload trapping molecule and instructions for use.

35. A pharmaceutical composition comprising an article of manufacture comprising a particulate delivery system comprising a yeast cell wall particle, a payload trapping molecule and a payload molecule selected from the group consisting of a polynucleotide, a protein, a small organic molecule and a mixture thereof, and a pharmaceutically acceptable excipient.

36. A method of delivering a payload molecule to a cell comprising the steps of:

providing an extracted yeast cell wall comprising beta-glucan, the yeast cell wall defining an internal space;

contacting the extracted yeast cell wall with a payload molecule wherein the payload molecule becomes at least partially enclosed within the internal space;

contacting the extracted yeast cell wall with a payload trapping molecule wherein the payload trapping molecule at least partially confines the payload molecule within the extracted yeast cell wall to form a particulate delivery system, wherein the payload molecule and the payload trapping molecule are soluble in the same solvent system; and

contacting a cell with the particulate delivery system.

37. The method of claim 36 further comprising the step of internalizing the particulate delivery system by the cell.

38. The method of claim 36 wherein the extracted yeast cell wall further comprises less than 90 weight percent beta-glucan.

39. The method of claim 36 wherein the extracted yeast cell wall further comprises more than 50 weight percent chitin.

40. The method of claim 36 wherein the extracted yeast cell wall further comprises more than 30 weight percent mannan.

41. The method of claim 36 wherein the extracted yeast cell wall further comprises more than 1 weight percent protein.

42. The method of claim 36 wherein the payload trapping molecule is a polysaccharide selected from the group consisting of agarose, an alginate, a xanthan, a dextran, a chitosan, a galactomannan gum, a derivative thereof and a mixture thereof.

43. The method of claim 36 wherein the payload trapping molecule is polyacrylamide.

44. The method of claim 36 wherein the payload trapping molecule is a polyamide.

45. The method of claim 36 wherein the payload trapping molecule is selected from the group consisting of a cationic polymer, an anionic polymer, a cationic detergent, an anionic detergent and a mixture thereof.

46. The method of claim 45 wherein the cationic polymer is selected from the group consisting of chitosan, polyethylenimine and poly-L-lysine.

47. The method of claim 45 wherein the payload trapping molecule is a mixture of a cationic polymer and a cationic detergent.

48. The method of claim 45 wherein the cationic polymer is selected from the group consisting of a protein, a polypeptides, a short synthetic peptide, a helical amphiphilic peptide, a cationic dendrimers, glucaramide polymer, a N-substituted glycine oligomer, poly(2-methyl-acrylic acid 2-[(2-dimethylamino)-ethyl)-methyl-amino] -ethyl ester), poly(2-dimethylamino ethyl)- methacrylate and mixtures thereof.

49. The method of claim 45 wherein the anionic polymer is selected from the group consisting of alginate and xanthan.

50. The method of claim 45 wherein the cationic detergent is hexadecyltrimethylammoniumbromide.

51. The method of claim 36 wherein the payload trapping molecule is selected from the group consisting of a cationic polyelectrolyte, an anionic polyelectrolyte and an amphoteric polyelectrolyte.

52. The method of claim 51 wherein the cationic polyelectrolyte is selected from the group consisting of a copolymer of vinyl pyrrolidone and quaternary methyl methacrylate, a substituted polyacrylamide, polyethyleneimine, polypropyleneimine, a polyamine homopolymer, a polyamine co-polymer, polydiallyl dimethyl ammonium chloride, substituted dextrans; modified guar gum, a substituted protein, a polyamino acid, spermine and spermidine.

53. The method of claim 51 wherein the anionic polyelectrolyte is selected from the group consisting of a copolymer of methyl vinyl ether and maleic anhydride, a copolymer of methyl vinyl ether and maleic acid, alginic acid a carboxymethyl cellulose, a substituted polyacrylamide, a polyacrylic acid, a polystyrene sulfonic acid, a dextran sulphates, a substituted saccharide, heparin and pharmaceutically acceptable salts.

54. The method of claim 36 wherein the payload molecule is selected from the group consisting of a polynucleotide, a peptide, a protein, a small organic active agent, a small inorganic active agent and a mixture thereof.

55. The method of claim 54 wherein the polynucleotide is selected from the group consisting of an oligonucleotide, an antisense construct, a siRNA, an enzymatic RNA, a recombinant DNA construct and a mixture thereof.

56. The method of claim 55 wherein the recombinant DNA construct is an expression vector comprising a control element operatively linked to an open reading frame encoding a protein.

57. The method of claim 56 further comprising the step of expressing the protein.

58. The method of claim 57 wherein the protein encoded by the open reading frame is a structural protein, a protein having enzymatic activity, a membrane protein, a DNA binding protein or a signaling protein.

59. The method of claim 57 wherein the protein encoded by the open reading frame is an antigenic protein.

60. The method of claim 55 wherein the polynucleotide comprises a nucleotide sequence that restores the function of an absent, defective or inhibited gene.

61. The method of claim 57 wherein the protein encoded by the open reading frame is a protein that produces a therapeutic effect in an individual having a genetic disorder.

62. The method of claim 61 wherein the genetic disorder is Aarskog-Scott syndrome, Aase syndrome, achondroplasia, acrodysostosis, addiction, adreno-leukodystrophy, albinism, ablepharon-macrostomia syndrome, alagille syndrome, alkaptonuria, alpha-1 antitrypsin deficiency, Alport's syndrome, Alzheimer disease, asthma, autoimmune polyglandular syndrome, androgen insensitivity syndrome, Angelman syndrome, ataxia, ataxia telangiectasia, atherosclerosis, attention deficit hyperactivity disorder (ADHD), autism, baldness, Batten disease, Beckwith-Wiedemann syndrome, Best disease, bipolar disorder, brachydactyly, breast cancer, Burkitt lymphoma, chronic myeloid leukemia, Charcot-Marie-Tooth disease, Crohn's disease, cleft lip, Cockayne syndrome, Coffin Lowry syndrome, colon cancer, congenital adrenal hyperplasia, Cornelia de Lange syndrome, Costello syndrome, Cowden syndrome, craniofrontonasal dysplasia, Crigler-Najjar syndrome, Creutzfeldt-Jakob disease, cystic fibrosis, deafness, depression, diabetes, diastrophic dysplasia, DiGeorge syndrome, Down's syndrome, dyslexia, Duchenne

muscular dystrophy, Dubowitz syndrome, ectodermal dysplasia, Ellis-van Creveld syndrome, Ehlers-Danlos, epidermolysis bullosa, epilepsy, essential tremor, familial hypercholesterolemia, familial Mediterranean fever, fragile X syndrome, Friedreich's ataxia, Gaucher disease, glaucoma, glucose galactose malabsorption, glutaricaciduria, gyrate atrophy, Goldberg Shprintzen syndrome (velocardiofacial syndrome), Gorlin syndrome, Hailey-Hailey disease, hemihypertrophy, hemochromatosis, hemophilia, hereditary motor and sensory neuropathy (HMSN), hereditary non polyposis colorectal cancer (HNPCC), Huntington's disease, immunodeficiency with hyper-IgM, juvenile onset diabetes, Klinefelter's syndrome, Kabuki syndrome, Leigh's disease, long QT syndrome, lung cancer, malignant melanoma, manic depression, Marfan syndrome, Menkes syndrome, miscarriage, mucopolysaccharide disease, multiple endocrine neoplasia, multiple sclerosis, muscular dystrophy, myotrophic lateral sclerosis, myotonic dystrophy, neurofibromatosis, Niemann-Pick disease, Noonan syndrome, obesity, ovarian cancer, p53 tumor suppressor, pancreatic cancer, Parkinson disease, paroxysmal nocturnal hemoglobinuria, Pendred syndrome, peroneal muscular atrophy, phenylketonuria (PKU), polycystic kidney disease, Prader-Willi syndrome, primary biliary cirrhosis, prostate cancer, REAR syndrome, Refsum disease, retinitis pigmentosa, retinoblastoma, Rett syndrome, Sanfilippo syndrome, schizophrenia, severe combined immunodeficiency, sickle cell anemia, spina bifida, spinal muscular atrophy, spinocerebellar atrophy, SRY: sex determination, sudden adult death syndrome, Tangier disease, Tay-Sachs disease, thrombocytopenia absent radius syndrome, Townes-Brocks syndrome, tuberous sclerosis, Turner syndrome, Usher syndrome, von Hippel-Lindau syndrome, Waardenburg syndrome, Weaver syndrome, Werner syndrome, Williams syndrome, Wilson's disease, xeroderma pigmentosum or Zellweger syndrome.

63. The method of claim 54 wherein the protein is selected from the group consisting of growth hormone, prolactin, placental lactogen, erythropoietin, thrombopoietin, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-9, interleukin-10, interleukin-11, interleukin-12 (p35 subunit), interleukin-13, interleukin-15, oncostatin M, ciliary neurotrophic factor, leukemia inhibitory factor, alpha interferon, beta interferon, gamma interferon, omega interferon, tau interferon, granulocyte-colony stimulating factor, granulocyte-macrophage colony stimulating factor,

macrophage colony stimulating factor, cardiotrophin-1growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; factor VIIIC, factor IX tissue factor, von Willebrands factor, Protein C, atrial natriuretic factor, lung surfactant, urokinase, tissue-type plasminogen activator, bombazine, thrombin, alpha tumor necrosis factor, beta tumor necrosis factor, enkephalinase; RANTES, human macrophage inflammatory protein, serum albumin, mullerian-inhibiting substance, relaxin A-chain, relaxin B-chain, prorelaxin, mouse gonadotropin-associated peptide, DNase, inhibin, activin, vascular endothelial growth factor, a hormone receptor, a growth factors receptor, an integrin, protein A, protein D, a rheumatoid factor, a neurotrophic factor, bone-derived neurotrophic factor (BDNF), neurotrophin-3, neurotrophin-4, neurotrophin-5, or neurotrophin-6, NGF-beta, platelet-derived growth factor (PDGF); alpha fibroblast growth factor, beta alpha fibroblast growth factor, epidermal growth factor, transforming growth factor-alpha, transforming growth factor-beta1, transforming growth factor-beta2, transforming growth factor-beta3, transforming growth factor-beta4, transforming growth factor-beta5, insulin-like growth factor-I, insulin-like growth factor-II, des(1-3)-insulin-like growth factor-I, a insulin-like growth factor binding protein, CD3, CD4, CD8, CD19, CD20, an osteoinductive factor, an immunotoxin, a bone morphogenetic protein, a T-cell receptor, surface membrane proteins, decay accelerating factor, a viral antigen, a transport protein, homing receptor, an addressin, a regulatory protein, an immunoadhesin, an antibody and biologically active fragments or variants thereof.

64. The method of claim 56 wherein the protein encoded by the open reading frame is selected from the group consisting of growth hormone, prolactin, placental lactogen, erythropoietin, thrombopoietin, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-9, interleukin-10, interleukin-11, interleukin-12 (p35 subunit), interleukin-13, interleukin-15, oncostatin M, ciliary neurotrophic factor, leukemia inhibitory factor, alpha interferon, beta interferon, gamma interferon, omega interferon, tau interferon, granulocyte-colony stimulating factor, granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor, cardiotrophin-1growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone;

lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; factor VIIIC, factor IX tissue factor, von Willebrands factor, Protein C, atrial natriuretic factor, lung surfactant, urokinase, tissue-type plasminogen activator, bombazine, thrombin, alpha tumor necrosis factor, beta tumor necrosis factor, enkephalinase; RANTES, human macrophage inflammatory protein, serum albumin, mullerian-inhibiting substance, relaxin A-chain, relaxin B-chain, prorelaxin, mouse gonadotropin-associated peptide, DNase, inhibin, activin, vascular endothelial growth factor, a hormone receptor, a growth factors receptor, an integrin, protein A, protein D, a rheumatoid factor, a neurotrophic factor, bone-derived neurotrophic factor (BDNF), neurotrophin-3, neurotrophin-4, neurotrophin-5, or neurotrophin-6, NGF-beta, platelet-derived growth factor (PDGF); alpha fibroblast growth factor, beta alpha fibroblast growth factor, epidermal growth factor, transforming growth factor-alpha, transforming growth factor-beta1, transforming growth factor-beta2, transforming growth factor-beta3, transforming growth factor-beta4, transforming growth factor-beta5, insulin-like growth factor-I, insulin-like growth factor-II, des(1-3)-insulin-like growth factor-I, a insulin-like growth factor binding protein, CD3, CD4, CD8, CD19, CD20, an osteoinductive factor, an immunotoxin, a bone morphogenetic protein, a T-cell receptor, surface membrane proteins, decay accelerating factor, a viral antigen, a transport protein, homing receptor, an addressin, a regulatory protein, an immunoadhesin, an antibody and biologically active fragments or variants thereof.

65. The method of claim 54 wherein the small organic active agent is an oligomer of heterocyclic polyamides that binds to the minor groove of double stranded DNA in a sequence specific manner.

66. The method of claim 54 wherein the small organic active agent is an oligomer having monomeric subunits selected from the group consisting of N-methylimidazole carboxamide, N-methylpyrrole carboxamide, beta-alanine and dimethylaminopripylamide.

67. The method of claim 54 wherein the small organic active agent is a contraceptive agent, a gastrointestinal therapeutic agent, a non-steroidal antifertility agent, a

parasympathomimetic agent, a psychotherapeutic agent, a major tranquilizer, a minor tranquilizer, a rhinological decongestant, a sedative-hypnotic, a steroid, a sulfonamide, a vaccine; a vitamin, a nutrient, an antimalarial, an anti-migraine agent, an anti-Parkinson agent, an anti-spasmodic, an anticholinergic agent, an antitussive, a bronchodilator, a cardiovascular agent; an anti-hypertensive agent, a coronary vasodilator, an organic nitrate, an alkaloid, an analgesic, a narcotic, an anti-cancer agent, an anti-convulsant, an anti-emetic, an anti-inflammatory agent, a cytotoxic drug or an antibiotic.

68. The method of claim 54 wherein the small organic active agent is an antibiotic selected from the group consisting of a cephalosporin, chloramphenical, gentamicin, kanamycin A, kanamycin B, a penicillin, ampicillin, streptomycin A, antimycin A, chloropamtheniol, metronidazole, oxytetracycline, penicillin G, a tetracycline and mixtures thereof.

69. A method of delivering a drug to a phagocytic cell comprising the steps of:

providing an extracted yeast cell wall comprising beta-glucan, the yeast cell wall defining an internal space;

contacting the extracted yeast cell wall with a payload molecule wherein the payload molecule becomes associated with extracted yeast cell wall;

contacting the extracted yeast cell wall with a payload trapping molecule wherein the payload trapping molecule stabilizes the association of the payload molecule and the extracted yeast cell wall to form a particulate delivery system; and wherein the payload molecule and the payload trapping molecule are soluble in the same solvent system; and

contacting a phagocytic cell with the particulate drug delivery system.

70. The method of claim 69 further comprising the step of internalizing the particulate drug delivery system by the phagocytic cell.

71. The method of claim 70 further comprising the step of transporting the particulate drug delivery system by the phagocytic cell.

72. The method of claim71 further comprising the step of releasing the drug from the particulate drug delivery system.

73. The method of claim 69 wherein the phagocytic cell is a macrophage, a M cell of a Peyer's patch, a monocyte, a neutrophil, a dendritic cell, a Langerhans cell, a Kupffer cell, an alveolar phagocyte, a peritoneal macrophage, a milk macrophage, a microglial cell, an eosinophil, a granulocytes, a mesengial phagocyte or a synovial A cell.

74. The method of claim 69 wherein the payload molecule is selected from the group consisting of a polynucleotide, a peptide, a protein, a small organic active agent, a small inorganic active agent and a mixture thereof.

75. A method of making a particulate delivery system comprising the steps of providing an extracted yeast cell wall comprising beta-glucan, the yeast cell wall defining an internal space;

contacting the extracted yeast cell wall with a payload molecule wherein the payload molecule becomes associated with extracted yeast cell wall; and

contacting the extracted yeast cell wall with a payload trapping molecule wherein the payload trapping molecule stabilizes the association of the payload molecule and the extracted yeast cell wall to form a particulate delivery system.

76. The method of claim 75 further comprising the steps of washing and drying the particulate delivery system.

77. The method of claim 75 wherein the payload molecule is at least partially within the internal space defined by the yeast cell wall.

78. The method of claim 75 wherein the stabilization of the association of the payload molecule occurs least partially within the internal space defined by the yeast cell wall.

79. The method of claim 75 wherein the payload molecule is selected from the group consisting of a polynucleotide, a peptide, a protein, a small organic active agent, a small inorganic active agent and a mixture thereof.

80. The method of claim 79 wherein the polynucleotide is selected from the group consisting of an oligonucleotide, an antisense construct, a siRNA, an enzymatic RNA, a recombinant DNA construct and a mixture thereof.

81. The method of claim 80 wherein the recombinant DNA construct is an expression vector comprising a control element operatively linked to an open reading frame encoding a protein.

82. The method of claim 79 wherein the peptide is selected from the group consisting of a hormone, a neurotransmitter, a neuromodulator, an active fragment of a signaling protein, an active fragment of a receptor, an active fragment of a enzyme and an active fragment of a nucleic acid binding protein.

83. The method of claim 79 wherein the protein is selected from the group consisting of an enzyme, a structural protein, a signaling protein, a nucleic acid binding protein and a transcription factor.

84. The method of exposing an individual to an antigen comprising the step of contacting a phagocytic cell of the individual with a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a payload trapping molecule and payload molecule, wherein the payload molecule is a polynucleotide comprising a control element operatively linked to an open reading frame encoding a peptide that can be controllably expressed in the cells of the individual.

85. The method of claim 84 wherein the peptide comprises at least an epitope identical to, or substantially similar to an antigenic epitope displayed on a pathogen.

86. The method of claim 84 wherein the peptide comprises at least an epitope identical to, or substantially similar to an antigenic epitope of a hyperproliferative disease-associated protein.

87. The method of claim 84 wherein the peptide comprises at least an epitope identical to, or substantially similar to an antigenic epitope of an autoimmune disease-associated protein.

88. The method of claim 84 wherein the peptide is a toxoid.

89. The method of claim 84 wherein the phagocytic cell is a macrophage, a M cell of a Peyer's patch, a monocyte, a neutrophil, a dendritic cell, a Langerhans cell, a Kupffer cell, an alveolar phagocyte, a peritoneal macrophage, a milk macrophage, a microglial cell, an eosinophil, a granulocyte, a mesengial phagocyte or a synovial A cell.

90. The method of exposing an individual to an antigen comprising the step of contacting a phagocytic cell of the individual with a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a payload trapping molecule and payload molecule, wherein the payload molecule is an antigenic molecule.

91. The method of claim 90 wherein the payload molecule is selected from the group consisting of a polynucleotide, a peptide, a protein, a small organic active agent, a small inorganic active agent and a mixture thereof.

92. The method of claim 90 wherein the payload molecule is a toxoid.

93. The method of claim 90 wherein the antigenic molecule comprises at least an epitope identical to, or substantially similar to an antigenic epitope of a hyperproliferative disease-associated protein.

94. The method of claim 90 wherein the antigenic molecule comprises at least an epitope identical to, or substantially similar to an antigenic epitope of a autoimmune disease-associated protein.

95. The method of claim 90 wherein the phagocytic cell is a macrophage, a M cell of a Peyer's patch, a monocyte, a neutrophil, a dendritic cell, a Langerhans cell, a Kupffer cell, an alveolar phagocyte, a peritoneal macrophage, a milk macrophage, a microglial cell, an eosinophil, a granulocyte, a mesengial phagocyte or a synovial A cell.

96. A method of delivering a drug to a macrophage cell comprising the steps of:
providing a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a drug and a payload trapping molecule; and
contacting a macrophage cell with the particulate drug delivery system.

97. The method of claim 96 further comprising the step of internalizing the particulate drug delivery system by the macrophage.

98. The method of claim 97 further comprising the step of transporting the particulate drug delivery system by the macrophage.

99. The method of claim 98 further comprising the step of delivering the particulate drug delivery system to a macrophage-attracting site.

100. The method of claim 99, wherein the macrophage-attracting site is a site of infection, inflammatory reaction, hypoxia or hyperplasia.

101. The method of claim 99, wherein the macrophage-attracting site is a tumor.

102. The method of claim 99 further comprising the step of releasing the drug from the particulate drug delivery system.

103. The method of claim 102 further comprising the step of releasing the drug into the extracellular space.

104. The method of claim 102 wherein the step of releasing the drug includes the steps of expressing a recombinant protein and secreting the protein into the extracellular space.

105. A method of immunizing an individual against a hyperproliferative disease comprising the step of contacting cells of said individual with a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a payload trapping molecule and a payload molecule that is a polynucleotide comprising a control element operatively linked to an open reading frame encoding a peptide that comprises an epitope identical to, or substantially similar to, an epitope displayed on a hyperproliferative disease-associated protein, wherein encoded peptide is capable of being expressed in the cells of the individual.

106. A method of treating an individual suffering from a genetic disease comprising the step of contacting cells of said individual with a particulate delivery system comprising an extracted yeast cell wall comprising beta-glucan, a payload trapping molecule and a payload molecule that is a polynucleotide thereby administering to the cells a polynucleotide that comprises a nucleotide sequence that restores the activity of an absent, defective or inhibited gene.

107. The method of claim 106 wherein the polynucleotide comprises a regulatory element operatively linked to an open reading frame encoding a protein that produces a therapeutic effect in the individual, the protein being capable of being expressed in said cells.

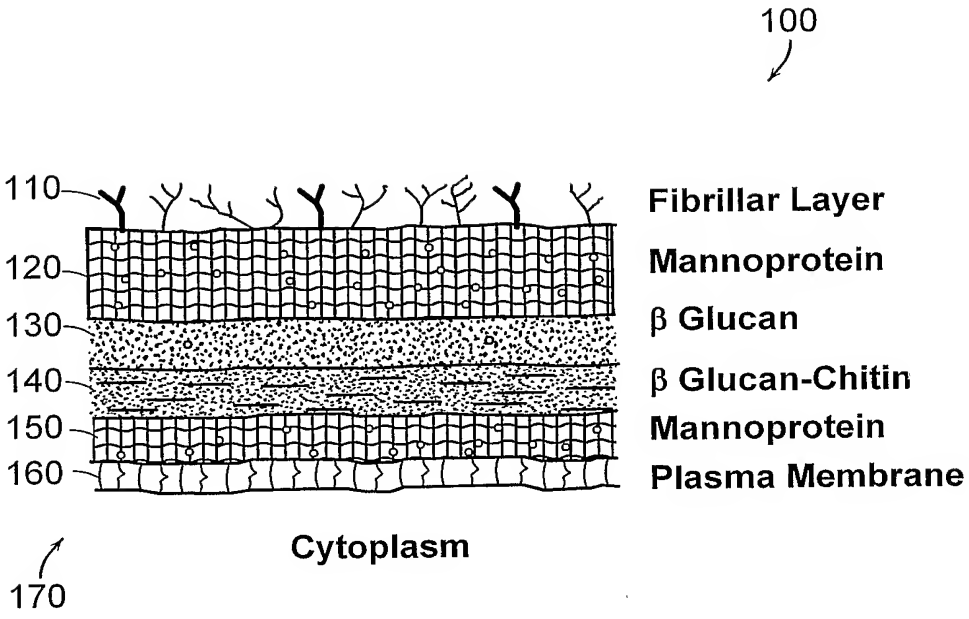

100
110
120
130
140
150
160
170
Fibrillar Layer
Mannoprotein
β Glucan
β Glucan-Chitin
Mannoprotein
Plasma Membrane
Cytoplasm

FIG. 1

Yeast Cell Wall Particle

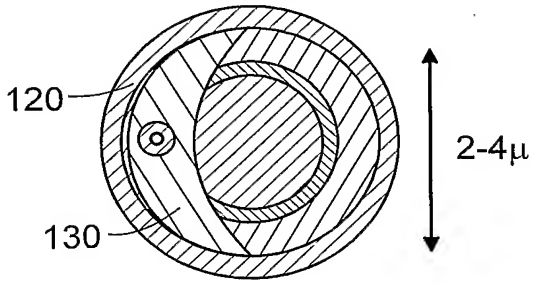


FIG. 2A

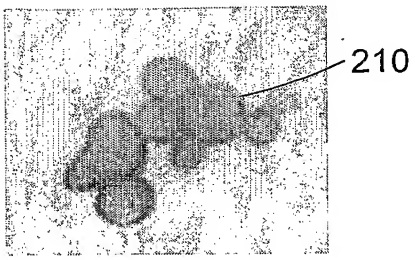


FIG. 2B

YGMP Beta Glucan

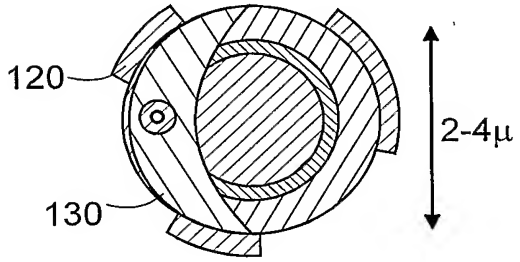


FIG. 2C

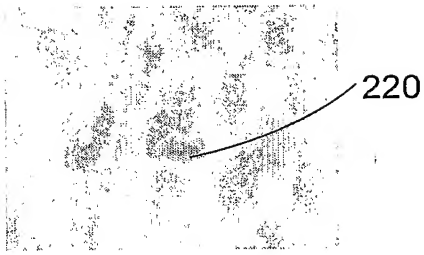


FIG. 2D

YGP Beta Glucan

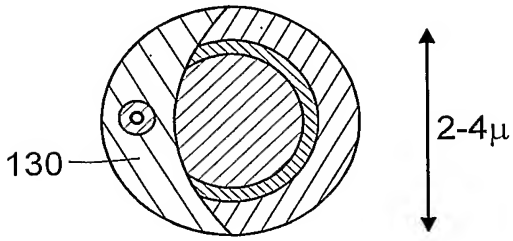


FIG. 2E

FIG. 2F

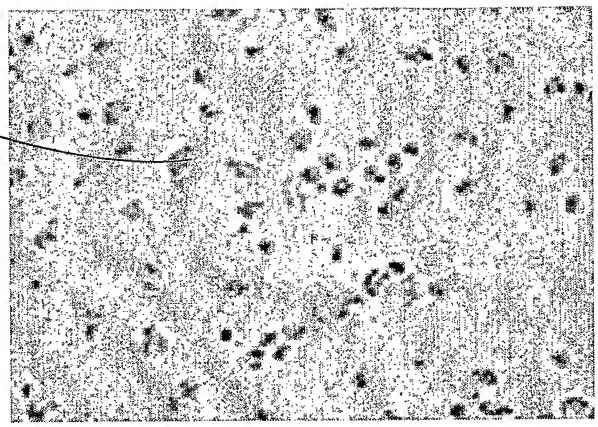


FIG. 3A

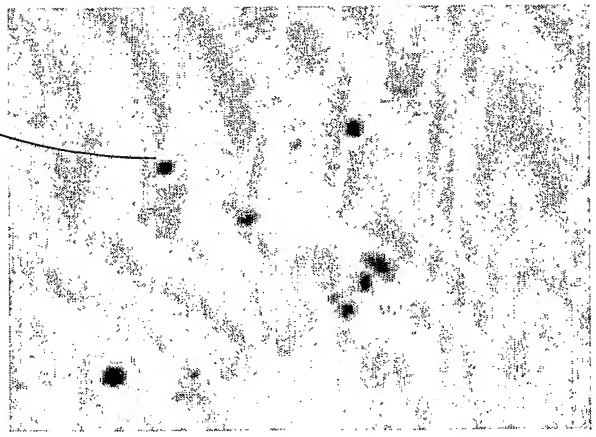
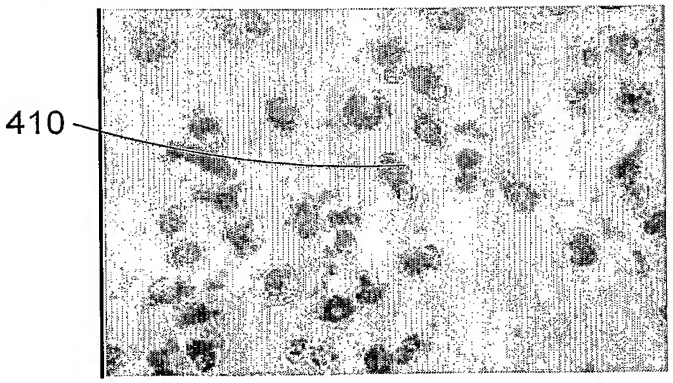


FIG. 3B



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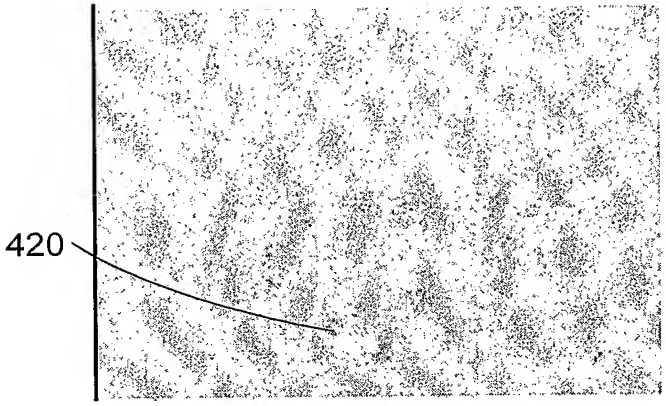

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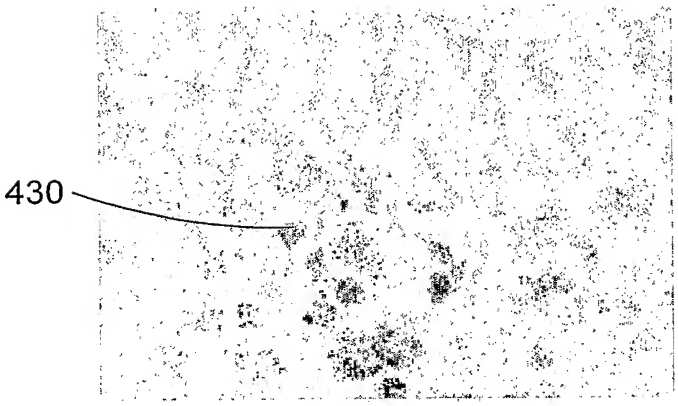

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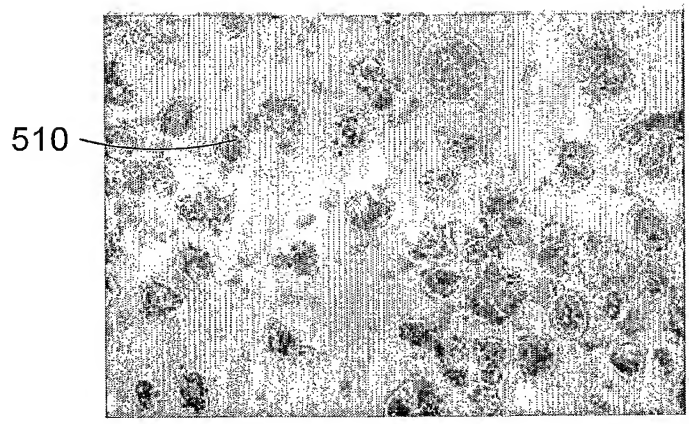
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FIG. 5A

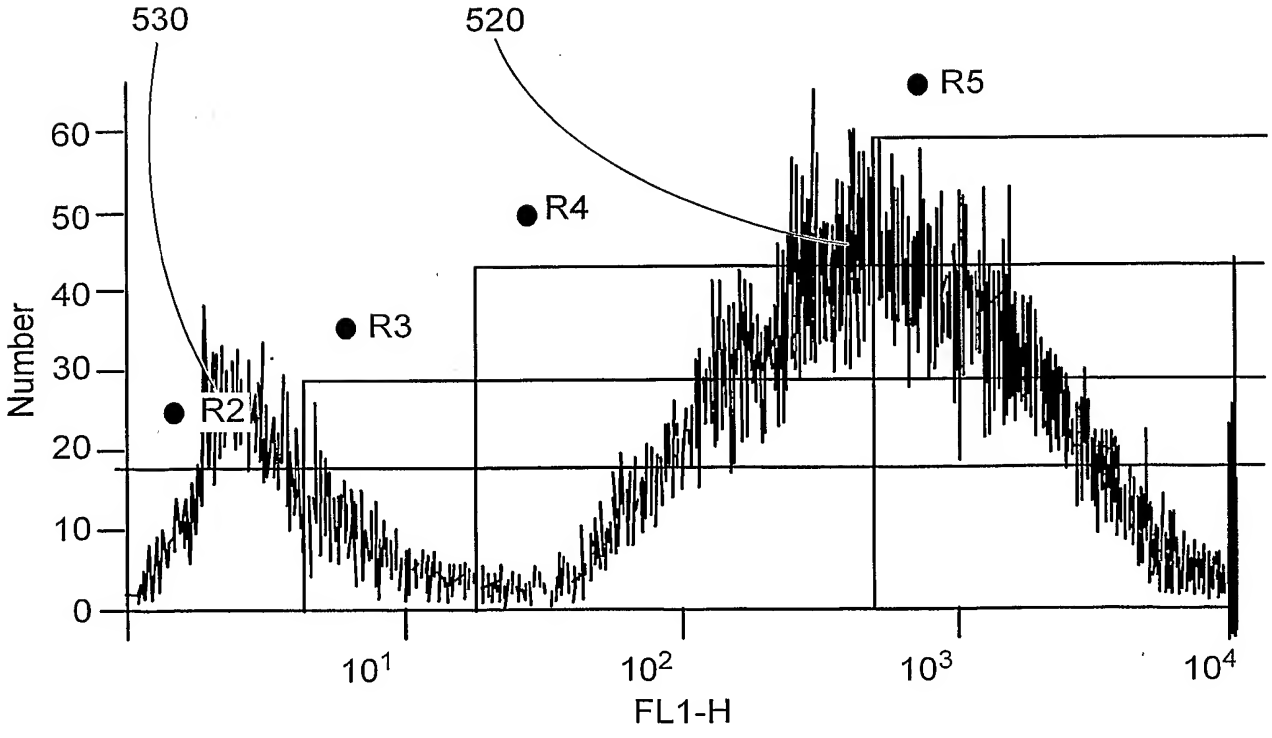
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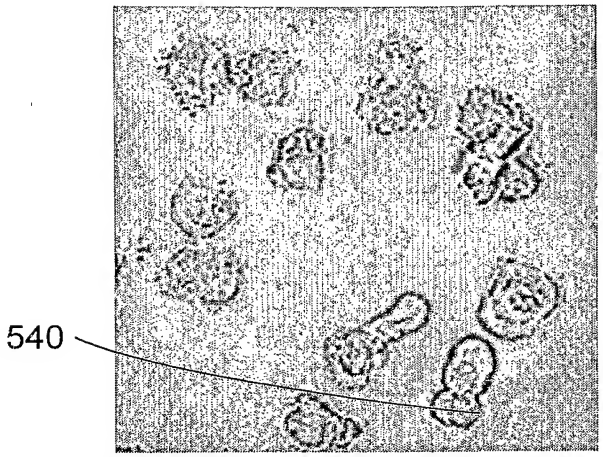
FIG. 5B



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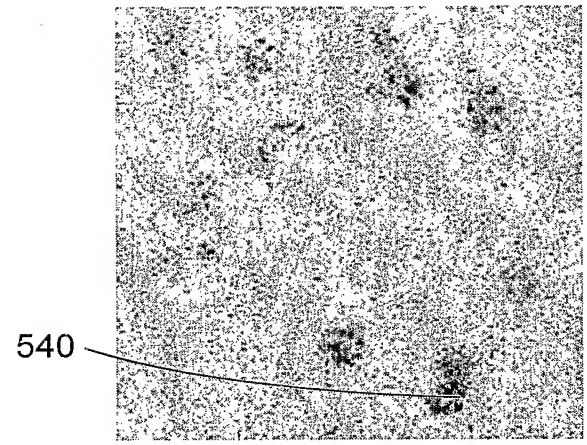

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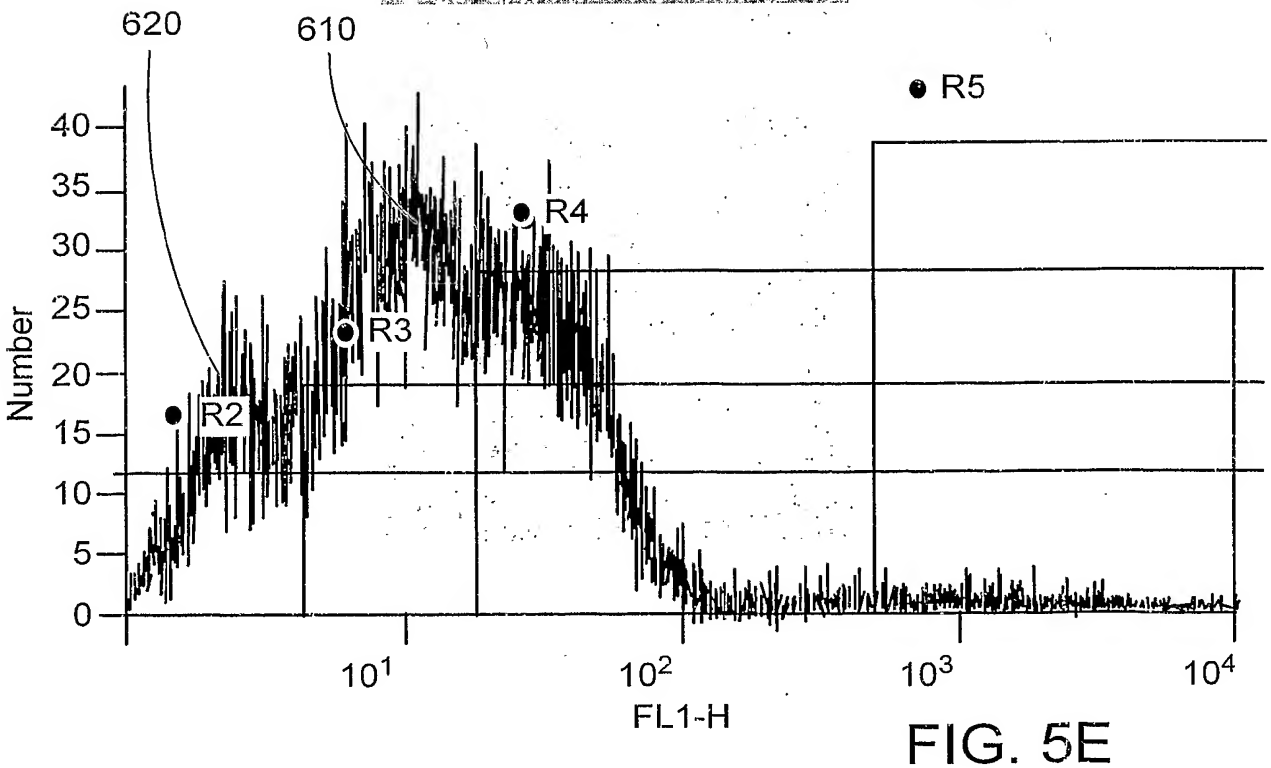

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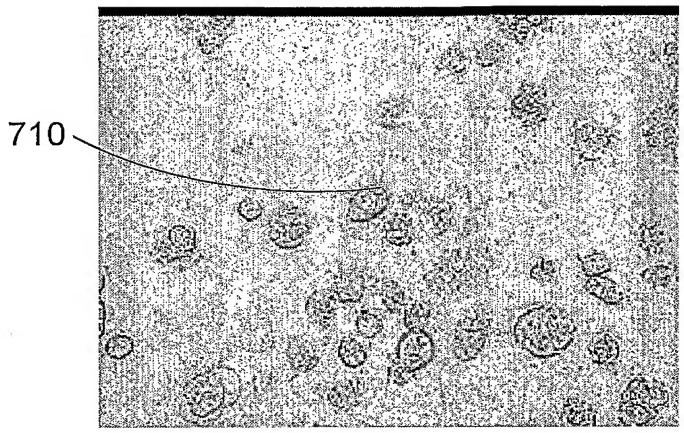

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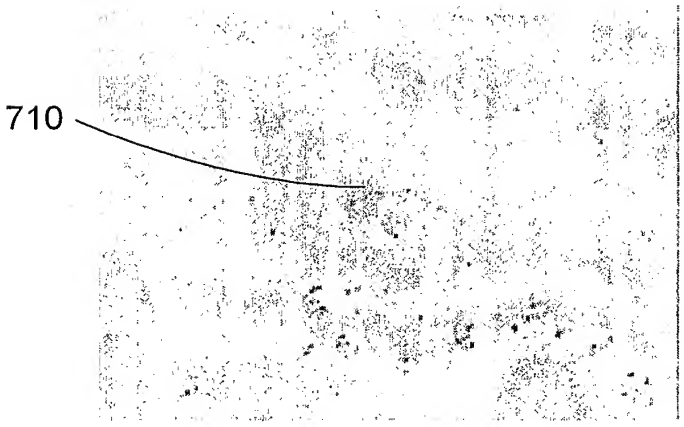

FIG. 5G

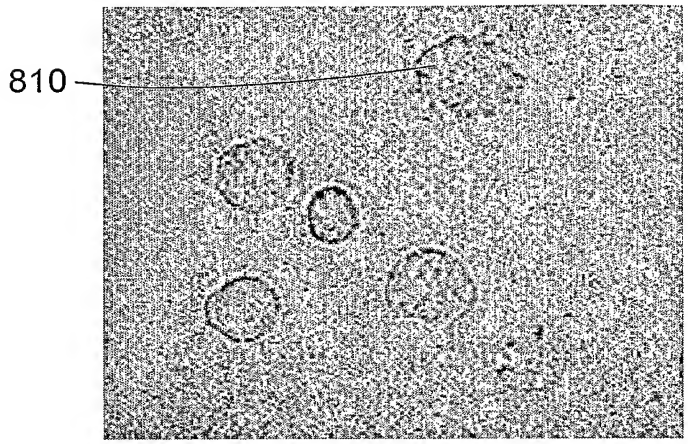

FIG. 5H

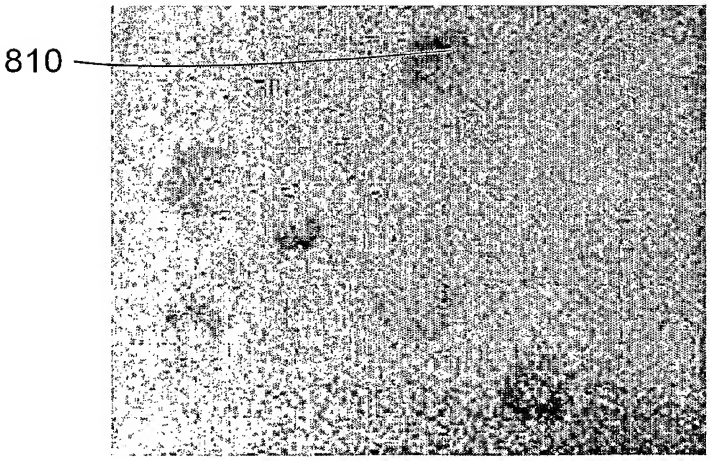

FIG. 5I

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Ostroff, Gary R.

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EXHIBIT 2

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European Patent Office
Office européen des brevets

(11) EP 0 759 089 B1

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(87) International publication number:
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(54) **ENZYME TREATMENT OF GLUCANS**

BEHANDLUNG VON GLUCANEN MIT ENZYMEN

TRAITEMENT ENZYMATIQUE DES GLUCANES

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26.02.1997 Bulletin 1997/09

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(56) References cited:
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- **DATABASE WPI Section Ch, Week 7949 Derwent Publications Ltd., London, GB; Class B04, AN 79-88295B & JP,A,54 138 115 (KIRIN BREWERY KK) , 26 October 1979**
- **FEBS LETTERS, vol. 64, no. 1, 1976 pages 44-47, S.BALINT ET AL. 'Biosynthesis of Beta-Glucans catalyzed by a particulate enzyme preparation from yeast'**
- **J. BIOCHEM., vol. 98, no. 5, 1985 pages 1301-1307, MASAO SHIOTA ET AL. 'Comparison of Beta-Glucan structures in a cell wall mutant of Sacch. cerevisiae and the wild type'**
- **DEV. COMP. IMMUN., vol. 18, no. 5, 1994 pages 397-408, R. ENGSTAD, B. ROBERTSEN 'Specificity of a Beta-Glucan Receptor on macrophages from atlantic salmon'**

EP 0 759 089 B1

Description

[0001] This invention relates to structurally modified yeast glucans, especially but not exclusively from the family Saccharomyces, obtainable by using β-(1-6)-glucanase. The use of such modified glucans in vaccine and animal feed formulations is described

Background of the Invention

[0002] It is known from European Patent Application No. 91111-143.3 (Publication No. 0466037 A2) that the immune system of aquatic animals can be stimulated through the administering of an effective amount of a yeast cell wall glucan. It is further known that the effect of vaccines on such aquatic animals can be enhanced by the administering of an effective amount of such yeast cell wall glucan along with the vaccine antigens.

[0003] Such glucan compositions are particulate glucans such as that derived from the yeast Saccharomyces cerevisiae. Such particulate glucans are macromolecular and are comprised of a chain of glucose units linked by β-(1-3)- and β-(1-6)-linkages, said glucan being a branced β-(1,3)-glucan having β-(1,3)-linked and β-(1,6)-linked chains therein.

[0004] Such particulate glucans are provided by KS Biotec-Mackzymal under the brand "MacroGard" and are potent activators of the macrophage/monocyte cell series. Thus such particulate glucans have a profound effect on the immune system.

[0005] While the particulate glucan derived from Saccharomyces cerevisiae is recognized to have a variety of beneficial effects on fish and other animals, the use of the glucan in the particulate and thus insoluble form is limited.

[0006] In addition it is now believed that the presence of β-(1-3)-branches contribute to the desired pharmaceutical benefits to be obtained from particulate glucan.

[0007] Accordingly a system whereby the of β-(1-3)-linked branches are made more readily available in the glucan would be highly desirable.

[0008] U.S. Patent No. 5,028,703 discloses and claims whole glucan particles wherein the β-(1-6) and β-(1-3)-linkage have been altered. Database WPI, Section ch, week 7949 class b04, AN 79-88295b discloses what is believed to be a glucan comprising mainly β-(1-3)-glucoside bonds since it is decomposed by β-(1-3)-glucanase.

[0009] Publication entitled *Specificity of Beta-glucan Receptor on Macrophages from Atlantic Salmon* from the Development and Comparative Immunology, vol. 18, no. 5, pp 397-408, 1994 provides an overview of a study of beta-glucan receptors in Atlantic salmon macrophages.

[0010] FEBS Letters, vol. 64, no. 1, 1976, pp 44-47 discloses the biosynthesis of β-glucans catalyzed by a particulate enzyme preparation of yeast.

SUMMARY OF THE INVENTION

[0011] It has been discovered that by treating the particulate glucan derived from yeast organisms, especially of the family Saccharomyces, and particularly Saccharomyces cerevisiae, with a β-(1-6)-glucanase, there is obtained a modified particulate glucan which is characterized by its enhanced activity in effecting stimulation of the immune system.

[0012] Thus in one embodiment of the present invention there is provided a novel β-(1-3)-glucan derived from yeast as defined in claims 1 and 5. As noted above, the yeast glucan is characterized by its enhanced ability to stimulate the immune system of fish and other animals.

[0013] In another embodiment of this invention there is provided a novel solubilized β-(1-3)-glucan from yeast which is useful for enhancing the activity of veterinary vaccines.

[0014] In still another embodiment of the present invention there is provided a novel feed grade glucan composition which is useful as an ingredient in conventional animal feeds.

[0015] Other embodiments and advantages of this invention will be apparent from the following specifications and claims.

Process for preparation of β-(1-6)-glucanase treated glucan ("MacroGard").

[0016] "MacroGard" brand glucan is derived from Saccharomyces cerevisiae as disclosed in European Application No. 91111143.3. While such glucan is known to stimulate the immune system of fish, it has been discovered that its activity is enhanced by the treatment thereof with a β-(1-6)-glucanase.

[0017] Such glucanase treatment of the glucan is carried out by suspending the glucan particle in a buffered medium at a pH in the range of about 4 to about 8 and at a temperature in the range of from about 20 to about 50°C. Suitable buffered media are those selected from the group consisting of sodium acetate, ammonium acetate and sodium-potassium phosphate. Presently preferred buffer solutions are sodium acetate or ammonium acetate. Enzymatic degradation of the glucan is commenced by the addition of the β-(1-6)-glucanase to the buffered medium.

[0018] β-(1-6)-glucanases which are suitable for the modification of yeast glucan are those obtained from a microorganism selected from the group consisting of Trichoderma longibrachiatum, Trichoderma reesei, Trichoderma harzianum, Rhizopus chinensis, Gibberella fujikuroi, Bacillus circulans, Mucor lilmalis, and Acineto-bacter. Of these a presently preferred glucanase is that obtained from Trichoderma harzianum.

[0019] The amount of β-(1-6)-glucanase employed for treatment of the glucan is normally in the range of from 1 to 50 U per g of glucan.

[0020] Enzymatic degradation is terminated by heating the reaction mixture to a temperature in the range of 80 to 100°C, preferably for a time in the range of 2 to 10 min. Other ways to stop the enzyme degradation are, e.g. by adding proteases or inhibitors to the reaction mixture.

[0021] Alternatively the enzyme may be simply removed by washing. The washed particles are then resuspended in water with the addition of a bactericide such as 0.3% formalin (v/v) and stored at a temperature of about 4°C.

[0022] The resulting enzyme treated glucan can be characterized as a branched β-(1-3)-glucan with β-(1-3)-linked sidechains being attached by a β-(1-6)-linkage and being essentially free of β-(1-6)-linked chains. In this connection the phraze "β(1-6) chains" is meant to include branches of more than 1 β(1-6)-linked glucose units. The β-(1-6)-glucanase enzyme cleavage ensures that most chains of more than 4 β-(1-6)-bound glucose units are cleaved off.

[0023] To further enhance the utility of the glucan, it is subject to solubilization. Such solubilization treatment is generally carried out at a temperature in the range of about from 70 to 90°C for a period of from about 30 to 60 min. in the presence of a solubilizing agent. A presently preferred solubilizing agent is formic acid. Following solubilization the solubilizing agent is removed and the resulting glucan is boiled in distilled water.

[0024] The glucan can be first enzyme treated and then solubilized or conversely be solubilized and then enzyme treated.

[0025] In accordance with another embodiment of this invention there is provided a β-(1-6)-glucanase treated feed grade glucan from yeast, e.g. Saccharomyces cerevisiae. Such feed grade glucan can be obtained by first contacting the yeast cell wall with an aqueous alkaline solution under conditions to effect the extraction of proteins and lipids therefrom. Generally such extraction is carried out at a temperature in the range from about 50 to 80°C for about 2 to 8 h. A presently preferred alkaline extraction agent is sodium hydroxide. Following extraction, the cell walls are recovered from the aqeous alkaline solution and washed to remove solubilized cell wall components therefrom. The washed yeast cell wall are then neutralized by treatment with an acid such as phosphoric acid. Thereafter the neutralized washed glucan is pasteurized and then dried.

[0026] Suitable enzymes for treatment of the feed grade glucan are those useful in treating the high purity glucan.

[0027] The enzyme treated feed grade glucan is prepared by contacting the glucan with a β-(1-6)-glucanase in the same manner as that employed for the enzyme treatment of the glucan particulate. The β-(1-6)-glucanase treated feed grade glucan of this invention is useful in animal feed formulations.

[0028] The following examples are presented for purposes of illustration of the invention.

EXAMPLE 1

[0029] This example provides the protocol used to obtain an immunostimulatory glucan particle suitable for utilization in the preparation of the yeast glucan of the present invention.

[0030] 500 g of dry Saccharomyces cerevisiae was suspended in 3 l of 6 % aqueous NaOH solution. This suspension was then stirred overnight at room temperature. After stirring the suspension was centrifuged at 2000 x g for 25 min. The supernatant was discarded and the insoluble residue was then resuspended in 3 l of 3 % NaOH and incubated for 3 h at 75°C followed by cooling the suspension overnight. The suspension was then centrifuged at 2000 x g for 25 min. and the supernatant was decanted. The residue was then resuspended in 3 % NaOH, heated and centrifuged as previously described.

[0031] The insoluble residue remaining was then adjusted to pH 4.5 with acetic acid. The insoluble residue was then washed with 2 l of water three times and recovered by centrifuging at 2000 x g for 25 min after each wash (the supernatant was poured off). The residue was then suspended in 3 l of a 0.5 M aqueous acetic acid. The suspension was heated for 3 h at 90°C. The suspension was then cooled to room temperature. After cooling, the insoluble residue was then collected by centrifuging at 2000 x g for 25 min. This treatment (from adjusting to pH 4.5 to collecting the cooled residue) was repeated 6 times.

[0032] The insoluble residue was then suspended in 3 l of distilled water and stirred for 30 min at 100°C, then cooled and centrifuged at 2000 x g for 25 min. The supernatant was discarded. The insoluble residue was washed in this manner 4 times. The residue was next suspended in 2 l of ethanol and heated at 78°C for 2 h. This wash with ethanol was repeated 4 times. The residue was then washed 4 times with 3 l of distilled water at room temperature to remove the ethanol, thereby providing a suspension of desired glucan product.

[0033] In more specific terms, the stepwise process for the production of the particulate β-(1-3)-glucan entails (a) alkali-extracting suitable glucan-containing yeast cells with a suitable extractive aqueous alkali solution under suitable

conditions to provide a first insoluble yeast residue; (b) hot alkali-extracting said first insoluble yeast residue with a suitable extractive aqueous alkali solution under suitable extraction conditions wherein the hot alkali extraction is performed at least 2 times to provide a second insoluble yeast residue and recovering the insoluble yeast residue after hot alkali extraction; thereafter (c) washing said second insoluble yeast residue with a suitable hydrolyzing acid under suitable conditions with water at a pH in the range of from about pH 4 to about pH 7 thereby providing a third insoluble yeast residue and recovering said third insoluble yeast residue after the wash; (d) hydrolyzing said third insoluble yeast residue under mild acidic hydrolysis condition wherein the acid hydrolysis is performed at lest 3 times to provide a fourth insoluble yeast residue and recovering the yeast residue after each hydrolysis; thereafter (e) boiling said fourth insoluble yeast residue under suitable conditions in water wherein the boiling of said fourth insoluble yeast residue is performed at least 2 times to provide a fifth insoluble °yeast residue and recovering the insoluble yeast residue after each boiling; and (f) boiling said fifth insoluble yeast residue under suitable conditions in ethanol wherein the boiling in ethanol of said fifth yeast residue is performed at least 2 times to provide a sixth insoluble yeast residue and recovering the insoluble yeast residue after each boiling; thereafter (g) washing said sixth insoluble yeast residue under suitable conditions with water wherein the washing of said sixth yeast residue is performed at least 2 times to provide a yeast glucan and recovering the insoluble yeast residue after each wash.

EXAMPLE 2

[0034] This example provides the protocol to obtain glucan particles essentially free of β-(1-6)-linked chains with the use of β-(1-6)-glucanase isolated from Trichoderma harzianum.

[0035] 200 mg of glucan particles prepared in accordance with the Example 1 were suspended in 40 ml 50 mM ammonium acetate buffer, pH 5.0, together with 10 U of β-(1-6)-glucanase at 37°C for 6h with constant stirring. The enzymatic degradation of the glucan particles was ended by heating the suspension at 100°C for 5 min. The particles were then washed three times with 200 ml sterile distilled H_2O by centrifugation at 2000 x g for 10 min, whereafter 185 mg of dried enzyme treated glucan was obtained.

[0036] The enzyme treatment will only cleave β-(1-6)-linkages within β-(1-6)-linked chains, but will not remove the β-(1-6)-linked glucosyl residue extending from the branching points. The resulting enzyme treated glucan can be characterized as a branched β-(1-3)-glucan with β-(1-3)-linked sidechains being attached by a β-(1-6)-linkage and being essentially free of β-(1-6)-linked chains, i.e. being free of β-(1-6)-linked chains apart from those chains of 4 or less β-(1-6)-bound glucose units.

EXAMPLE 3

[0037] This example provides the protocol to solubilize glucan particles prepared in accordance with Example 1 by hydrolysis using formic acid (HCOOH).

[0038] 2.0 g of glucan particles were suspended in 1.0 l of 90% formic acid and heated at 80°C for 45 min under constant stirring. The suspension was cooled to 35°C and the formic acid was evaporated. The residue containing the hydrolysed particles was boiled in 500 ml distilled water for 3 h, whereafter the cooled suspension was filtrated through a 0.44 μm filter, frozen and lyophilized whereby 1.9 g of dry solubilized particles were obtained. The lyophilized solubilized particles were then dissolved in 100 ml distilled water and dialyzed, using a tubular dialysis membrane having a nominal molecular weight cut off (NMWCO) of 5000 Dalton, against tap water for 24 h, and then lyophilized. This resulted in 1.8 g solubilized glucan product.

EXAMPLE 4

[0039] This example demonstrates the biological effects of glucan particles prepared according to Example 1, and β-(1-6)-glucanase treated glucan particles prepared according to Example 2 on immune responses in Atlantic salmon.

[0040] An A-layer positive isolate of Aeromonas salmonicida subsp. salmonicida, referred to as strain no. 3175/88 (Vikan Veterinary Fish Research Station, Namsos, Norway) was used. The bacterium was grown in brain heart infusion broth (Difco, USA) for 30 h at 14°C in a shaker incubator, and the culture medium with the bacterium was centrifuged for 10 min at 3000 x g. The pellet was resuspended in 0.9% saline, and the bacterium was killed by adding 0.5% formalin (v/v) to the suspension and incubating at 14°C for 24 h. The formalinized culture was then washed with sterile 0.9% saline and resuspended to a concentration of 2 x 10^9 ml^{-1} bacteria in 0.9% saline with 0.3% formalin. Bacterial suspensions ware mixed with an equal volume of saline or with the different glucan suspensions (10 mg ml^{-1} in saline). Formalin was added to the vaccines to a final concentration of 0.3% (v/v).

[0041] In carrying out these experiments, two groups of experimental fish were used. In the vaccine experiment, Atlantic salmon presmolts of 20 - 40 g were used. In the experiment where serum was collected for measuring blood lysozyme activity after glucan injection, Atlantic salmons of 50 - 70 g were used. The fish were kept in 150 l tanks

supplied with aerated fresh water at 12°C and fed with commercial pellets ad libitum twice daily.

[0042] In the vaccination experiment 40 fish in each group were IP-injected with 0.1 ml of the different vaccine preparations or vaccine without glucan as a control. Blood was collected in evacuated tubes (Venoject, Terumo-Europe, Belgium) from 10 fish in each group 6, 10, and 18 weeks after injection. Blood samples were allowed to clot overnight at 4°C and sera were collected after centrifuging the tubes at 2000 x g for 10 min. Individual serum samples were transferred to Micronic serum tubes (Flow Laboratories Ltd., Lugano, Switzerland) and stored at -80°C until required.

[0043] In order to measure the effect of glucans on blood lysozyme activity, salmons were IP injected with 0.3 ml of the different glucans in saline or with 0.3 ml saline as the negative control. The glucans were administered at a concentration of 10 mg ml^{-1}. Blood samples were collected from 10 fish from each group 10 and 20 days after injection, using evacuated tubes (Venoject). The tubes were kept on ice until centrifuged at 2000 x g for 10 min, and individual serum samples were transferred to Micronic serum tubes and stored at -80°C until required.

[0044] Lysozyme activity was measured with the turbidimetric method using 0.2 mg ml^{-1} lyophilized Micrococcus lysodeikticus as the substrate in 0.04 M sodium phosphate buffer at pH 5.75. Serum (20 µl) was added to 3 ml of the suspension and the reduction in absorbance at 540 nm was measured after 0.5 min and 4.5 min at 22°C. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}. Results are expressed as mean lysozyme activity in serum from 10 fish (Tables 1 and 2).

[0045] The level of specific antibody against the A-layer of A. salmo-nicida in salmon sera was measured by an enzyme-linked immunosorbent assay (ELISA). A-layer protein was purified from whole A. salmonicida cells (Bjørnsdottir et al. (1992), Journal of Fish Diseases, 15:105-118), and protein content was determined (Bradford, M.M. (1976), Analytical Biochemistry, 72:248-254) using a dye-reagent concentrate from Bio-Rad Laboratories (Richmont, USA). Microtitre plates were coated with 100 µl of 5 µg ml^{-1} A-layer protein in 50 mM carbonate buffer, pH 9.6, and incubated overnight at 4°C. The further procedure was performed as described by Havardstein et al. (Journal of Fish Diseases (1990), 13:101-111). The antibody titre in pooled serum samples was determined before individual serum samples were measured at three different dilutions (1:500, 1:1000 and 1:2000). Absorbance was read at 492 nm in a Multiscan MCC/340 MK II (Flow Laboratories Ltd). Results are expressed as mean antibody response to the A-layer of the bacterium at a dilution of 1:2000 in serum from 10 fish (Tables 1 and 2).

Table 1.

Differences in biological effects of glucan particles and β-(1-6)-glucanase treated glucan particles on immune responses in Atlantic salmon.			
	Saline control	Untreated glucan particles	β-(1,6)-glucanase treated glucan particles
Lysozyme activity post injection (units/ml)			
10 days	304	505	529
20 days	330	407	454
	Vaccine without glucan	Vaccine with untreated glucan particles	Vaccine with β-(1,6)-glucanase treated glucan particles
Antibody response post injection (absorbance)			
6 weeks	0.165	0.255	0.376
10 weeks	0.059	0.355	0.500
18 weeks	0.037	0.197	0.142

[0046] Both injection of untreated and β-(1,6)-glucanase treated glucan particles induced significantly higher ($p<0.01$) lysozyme activity in serum compared to saline control both 10 and 20 days post injection. At day 20 post injection the lysozyme levels in fish injected with β-(1,6)-glucanase treated glucan particles were significantly higher ($p<0.05$) compared to fish injected with untreated particles.

[0047] β-(1,6)-glucanase treated glucan particles induced significantly higher ($p<0.05$) antibody response to the vaccine compared to vaccine without adjuvant at all three sampling times, whereas untreated glucan particles induced significantly higher response 10 and 18 weeks post injection. β-(1,6)-glucanase treated glucan particles induced significantly higher ($p<0.05$) antibody response than did untreated glucan particles at 10 weeks post injection, whereas no significant differences between the two were observed at 6 and 18 weeks post injection.

Table 2.

Biological effects of glucan particles and solubilized glucan.			
	Saline control	Untreated glucan particles	Solubilized glucan particles
Lysozyme activity (units/ml)			
10 days after injection	304	505	603
20 days after injection	330	407	773
	Vaccine without glucan	Vaccine with untreated glucan particles	Vaccine with solubilized glucan particles
Adjuvant effect (absorbance)			
6 weeks after injection	0.165	0.255	0.184
10 weeks after injection	0.059	0.355	0.349
18 weeks after injection	0.037	0.197	0.120

[0048] Injection of solubilized glucan particles induced significant higher ($p<0.01$) lysozyme activity than did untreated glucan particles both 10 and 20 days post injection. No significant differences could be observed between the ability of solubilized glucan particles and untreated glucan particles to induce increased antibody response to the vaccine antigen at any sampling time point. Both induced significant higher ($p<0.05$) antibody response than vaccine without adjuvant 10 and 18 weeks post injection, but not at 6 weeks post injection.

EXAMPLE 5

[0049] This example provides the protocol to obtain a glucan composition suitable for use in the feeding of animals.

[0050] 1000 kg of dry cell wall material of Saccharomyces cerevisiae was suspended in 5300 l of water at a temperature of 65°C in a stainless steel tank. To the suspension of cell walls in water there was added 227 l of 50% w/w NaOH so as to provide a caustic concentration of about 3%. The resulting mixture was then stirred for a period of about 4 h at a temperature of about 60°C.

[0051] Following the initial extraction period the suspension was then diluted with 8000 kg of water at a temperature of about 65°C in a stainless steel, stirred, washing tank such that the weight of the mixture was doubled. The resulting diluted mixture was then maintained at a temperature of about 60°C while being stirred for a period of about 15 min. Thereafter the resulting mixed diluted suspension was centrifuged in a nozzle centrifuge (Alfa Laval DX209). The supernatant was discarded. The resulting concentrated cell wall suspension was continuously introduced into a second steel stirred wash tank containing 8000 kg water and the mixture adjusted by the addition of water to give a final weight of 14500 kg. The resulting suspension was then mixed for a period of 15 min at a temperature of 60-65°C. Thereafter the agitated mixture was centrifuged.

[0052] The resulting cell wall suspension was continuously added to a third vessel containing 8000 kg water. Additional water at 60°C was added to provide a final weight of 14500 kg. The resulting suspension was stirred for a period of 15 min at 60-65°C and thereafter centrifuged.

[0053] Following centrifugation, the resulting cell wall concentrate was transferred to a stainless steel storage tank wherein the suspension.was cooled to a temperature of about 5-10°C. The resulting cooled suspension was treated with phosphoric acid (H_3PO_4) in a stainless steel agitated tank in an amount to achieve a suspension of solids having a pH of 5.5-7.5.

[0054] Following neutralization the resulting neutralized mixture was subjected to pasteurization by heating at a temperature of 75°C for a period of 18 seconds by passing the mixture through an in-line plate and frame heat exchanger.

[0055] Following pasteurization the resulting pasteurized mixture was then spray dried in a spray drier maintained at an inlet air temperature of at least 140-150°C and an exhaust temperature of about 65-70°C whereby there was achieved 300 kg of dry glucan product.

EXAMPLE 6

[0056] This example provides the protocol and effect of treatment of feed grade glucan with a β-(1-6)-glucanase.

[0057] 25 g of feed grade glucan, prepared in accordance with Example 5, suspended in 1.25 l of 50 mM sodium acetate, pH 5.0, in a 2 l conical flask. Glucan particles were maintained in suspension by shaking, the suspension was warmed to 30°C and purified β-(1-6)-glucanase from Trichoderma harzianum was added to a final concentration of 1.8 U/g glucan.

[0058] To follow the timecourse of the enzymatic removal of β-1,6-bound glucose from the glucan particle, the suspension were withdrawn at different timepoints, centrifuged at 2000 x g, and 0.2 ml of the supernatants analyzed for free, reducing carbohydrate (Nelson et al. (1944), Journal of Biological Chemistry, (153:315-80). The glucan suspension was incubated for 28h at which time the rate of release of free, reducing carbohydrate was observed to be very low. The glucan particles were then pelleted by centrifugation at 2000 x g, washed once in 50 mM sodium-acetate, pH 5.0 and once in water.

[0059] A fine, dry powder suitable for use as a feed additive was prepared from the wet glucan by first dehydrating the pellet four times with ethanol at room temperature followed by air drying at room temperature.

[0060] The stepwise process for the production of the feed grade glucan includes (a) contacting yeast cell walls with an aqueous alkaline solution under suitable conditions to effect the extraction of proteins and lipids therefrom; (b) separating the resulting extracted yeast cell walls from said aqueous alkaline solution; (c) washing the resulting separate yeast cells so as to further remove solubilized cell wall components therefrom; (d) neutralizing the washed yeast cell walls; and (e) pasteurizing the neutralized, washed cell walls and thereafter drying the resulting pasteurized, neutralized, washed cell walls.

[0061] Results from treating a feed grade glucan with β-(1-6)-glucanase from T. harzianum as described above are shown in Table 3.

Table 3.

Liberation of glucose from feed grade glucan during treatment with β-(1-6)-glucanase from T. harzianum.	
Enzyme reaction time, [h]	Glucose liberated, [% of total glucose in glucan]
0	0.0
0.5	1.9
1	2.6
2	3.3
3	3.7
4	4.0
5	4.3
2	5.5
8	5.6

Claims

1. A branched β-(1-3)-glucan with β-(1-3)-linked sidechains being attached by a β-(1-6)-linkage and being free of β-(1-6)-linked chains apart from those chains of 4 or less β-(1-6)-bound gluclose units, said glucan being obtainable by the process of contacting an insoluble, particulate, branched β-(1-3)-glucan derived from yeast having β-(1-3)-linked and β-(1-6)-linked chains therein with a β-(1-6)-glucanase.

2. The glucan of claim 1, wherein said insoluble, particulate, branched β-(1-3)-glucan having β-(1-3)-linked and β-(1-6)-linked chains therein is prepared by the process comprising:

   (a) alkali-extracting suitable glucan-containing yeast cells with a suitable extractive aqueous alkali solution under suitable conditions to provide a first insoluble yeast residue;
   (b) hot alkali-extracting said first insoluble yeast residue with a suitable extractive aqueous alkali solution under suitable extractive conditions, wherein the hot alkali extraction is performed at least 2 times to provide a second insoluble yeast residue, and recovering the insoluble yeast residue after hot alkali extraction; thereafter
   (c) washing said second insoluble yeast residue with a suitable hydrolyzing acid under suitable conditions with water at a pH in the range of about pH 4 to about pH 7, thereby providing a third insoluble yeast residue, and recovering said third insoluble yeast residue after the wash;
   (d) hydrolyzing said third insoluble yeast residue under mild acidic hydrolysis condition, wherein the acid hydrolysis is performed at least 3 times to provide a fourth insoluble yeast residue, and recovering the yeast residue after each acid hydrolysis; thereafter

(e) boiling said fourth insoluble yeast residue under suitable conditions in water, wherein the boiling of said fourth insoluble yeast residue is performed at least 2 times to provide a fifth insoluble yeast residue, and recovering the insoluble yeast residue after each boiling; and
(f) boiling said fifth insoluble yeast residue under suitable conditions in ethanol, wherein the boiling in ethanol of said fifth yeast residue is performed at least 2 times to provide a sixth insoluble yeast residue, and recovering the insoluble yeast residue after each boiling; thereafter
(g) washing said sixth insoluble yeast residue under suitable conditions with water, wherein the washing of said sixth yeast residue is performed at least 2 times to provide a yeast glucan, and recovering the insoluble yeast residue after each wash.

3. A solublized β-(1-3)-glucan with β-(1-3)-linked sidechains being attached by a β-(1-6)-linkage and being free of β-(1-6)-linked chains apart from those chains of 4 or less β-(1-6)-bound glucose units, said glucan being obtainable by the process of contacting a branched glucan product of claim 1 or claim 2 with a solubilizing agent.

4. The solubilized glucan product of claim 3, wherein said solubilizing agent is formic acid and said branched glucan is contacted with said solubilizing agent at a temperature in the range of from 70 to 90 °C.

5. An insoluble, particulate yeast glucan, especially from the yeast family Saccharomyces, and particularly from the yeast species Saccharomyces cerevisiae, being **characterized** as a branched β-(1-3)-glucan with β-(1-3)-linked sidechains being attached by a β-(1-6)-linkage and being free of β-(1-6)-linked chains apart from those chains of 4 or less β-(1-6)-bound glucose units.

6. A branched β-(1-3)-feed grade yeast glucan with β-(1-3)-linked sidechains being attached by a β-(1-6)-linkage and being free of β-(1-6)-linked chains apart from those chains of 4 or less β-(1-6)-bound glucose units, said glucan being obtainable by the process of contacting an insoluble, particulate, branched β-(1-3)-feed grade glucan derived from yeast having β-(1-3)-linked and β-(1-6)-linked chains therein with a β-(1-6)-glucanase.

7. The feed grade yeast glucan of claim 6, wherein said insoluble, particulate, branched β-(1-3)-feed grade glucan having β-(1-3)-linked and β-(1-6)-linked chains therein is prepared by the process comprising:

(a) contacting yeast cell walls with an aqueous alkaline solution under suitable conditions to effect the extraction of proteins and lipids therefrom;
(b) separating the resulting extracted yeast cell walls from said aqueous alkaline solution;
(c) washing the resulting separate yeast cells so as to further remove solubilized cell wall components therefrom;
(d) neutralizing the washed yeast cell walls; and
(e) pasteurizing the neutralized, washed cell walls and thereafter drying the resulting pasteurized, neutralized, washed cell walls.

Patentansprüche

1. Verzweigtes β-(1-3)-Glucan mit β-(1-3)-verknüpften Seitenketten, die über eine β-(1-6)-Verknüpfung angelagert sind und die frei sind von β-(1-6)-verknüpften Ketten außer solchen Ketten von 4 oder weniger β-(1-6)-gebundenen Glucose-Einheiten, welches Glucan erhalten werden kann mit Hilfe des Verfahrens des Kontaktierens eines unlöslichen, partikulären verzweigten β-(1-3)-Glucans, deriviert von Hefe, mit β-(1-3)-verknüpften und β-(1-6)-verknüpften Ketten darin, mit einer β-(1-6)-Glucanase.

2. Glucan nach Anspruch 1, bei welchem das unlösliche, partikuläre verzweigte β-(1-3)-Glucan mit β-(1-3)-verknüpften und β-(1-6)-verknüpften Ketten darin hergestellt wird nach dem Verfahren, umfassend:

(a) alkalisches Extrahieren geeigneter, Glucan enthaltender Hefezellen mit einer geeigneten extrahierenden wässrigen Alkalilösung unter geeigneten Bedingungen, um einen ersten unlöslichen Heferückstand zu schaffen;
(b) heißes alkalisches Extrahieren des ersten unlöslichen Heferückstands mit einer geeigneten extrahierenden wässrigen Alkalilösung unter geeigneten Extraktionsbedingungen, wobei das heiße alkalische Extrahieren mindestens zweimal ausgeführt wird, um einen zweiten unlöslichen Heferückstand zu schaffen, und Gewinnen des unlöslichen Heferückstands nach der heißen alkalischen Extraktion; und danach

(c) Waschen des zweiten unlöslichen Heferückstands mit einer geeigneten hydrolysierenden Säure unter geeigneten Bedingungen mit Wasser im Bereich von etwa pH 4 bis etwa pH 7, womit ein dritter unlöslicher Heferückstand geschaffen wird sowie Gewinnen des dritten unlöslichen Heferückstands nach der Wäsche;
(d) Hydrolysieren des dritten unlöslichen Heferückstands unter Bedingungen einer milden sauren Hydrolyse, wobei die saure Hydrolyse mindestens dreimal ausgeführt wird, um einen vierten unlöslichen Heferückstand zu schaffen, und Gewinnen des Heferückstands nach jeder sauren Hydrolyse; und danach
(e) Kochen des vierten unlöslichen Heferückstands unter geeigneten Bedingungen in Wasser, wobei das Kochen des vierten unlöslichen Heferückstands mindestens zweimal ausgeführt wird, um einen fünften unlöslichen Heferückstand zu schaffen, und Gewinnen des unlöslichen Heferückstands nach jedem Kochen; und
(f) Kochen des fünften unlöslichen Heferückstands unter geeigneten Bedingungen in Ethanol, wobei das Kochen des fünften unlöslichen Heferückstands in Ethanol mindestens zweimal ausgeführt wird, um einen sechsten unlöslichen Heferückstand zu schaffen, und Gewinnen des unlöslichen Heferückstands nach jedem Kochen; und danach
(g) Waschen des sechsten unlöslichen Heferückstands unter geeigneten Bedingungen mit Wasser, wobei das Waschen des sechsten unlöslichen Heferückstands mindestens zweimal ausgeführt wird, um ein Hefeglucan zu schaffen, und Gewinnen des unlöslichen Heferückstands nach jeder Wäsche.

3. Solubilisiertes β-(1-3)-Glucan mit β-(1-3)-verknüpften Seitenketten, die über eine β-(1-6)-Verknüpfung angelagert sind und die frei sind von β-(1-6)-verknüpften Ketten außer solchen Ketten von 4 oder weniger β-(1-6)-gebundenen Glucose-Einheiten, welches Glucan erhalten werden kann mit Hilfe des Verfahrens des Kontaktierens eines verzweigten Glucan-Produkts nach Anspruch 1 oder Anspruch 2 mit einem Solubilisierungsmittel.

4. Solubilisiertes Glucan-Produkt nach Anspruch 3, wobei das Solubilisierungsmittel Ameisensäure ist und das verzweigte Glucan mit dem Solubilisierungsmittel bei einer Temperatur im Bereich von 70° bis 90°C kontaktiert wird.

5. Unlösliches, partikuläres Hefeglucan, speziell aus der Hefefamilie Saccharomyces und besonders von der Species Saccharomyces cerevisiae, **gekennzeichnet** als ein verzweigtes β-(1-3)-Glucan mit β-(1-3)-verknüpften Seitenketten, die über eine β-(1-6)-Verknüpfung angelagert sind und die frei sind von β-(1-6)-verknüpften Ketten außer solchen Ketten von 4 oder weniger β-(1-6)-gebundenen Glucose-Einheiten.

6. Verzweigtes β-(1-3)-Hefeglucan mit Futtermittelqualität mit β-(1-3)-verknüpften Seitenketten, die über eine β-(1-6)-Verknüpfung angelagert sind und die frei sind von β-(1-6)-verknüpften Ketten außer solchen Ketten von 4 oder weniger β-(1-6)-gebundenen Glucose-Einheiten, welches Glucan erhalten werden kann mit Hilfe des Verfahrens des Kontaktierens eines unlöslichen, partikulären verzweigten β-(1-3)-Hefeglucans mit Futtermittelqualität, deriviert von Hefe, mit β-(1-3)-verknüpften und β-(1-6)-verknüpften Ketten darin, mit einer β-(1-6)-Glucanase.

7. Hefeglucan mit Futtermittelqualität nach Anspruch 6, bei welchem das unlösliche, partikuläre verzweigte β-(1-3)-Hefeglucan mit Futtermittelqualität mit β-(1-3)-verknüpften und β-(1-6)-verknüpften Ketten darin hergestellt wird mit Hilfe des Verfahrens, umfassend:

(a) Kontaktieren von Hefezellwänden mit einer wässrigen alkalischen Lösung unter geeigneten Bedingungen, um die Extraktion von Proteinen und Lipiden daraus zu bewirken;
(b) Separieren der resultierenden extrahierten Hefezellwände aus der wässrigen alkalischen Lösung;
(c) Waschen der resultierenden separierten Hefezellen, um weitere solubilisierte Zellwand-Komponenten daraus zu entfernen;
(d) Neutralisieren der gewaschenen Hefezellwände; und
(e) Pasteurisieren der neutralisierten, gewaschenen Zellwände und danach Trocknen der resultierenden, pasteurisierten, neutralisierten, gewaschenen Zellwände.

Revendications

1. β-(1-3)-glucane ramifié à chaînes latérales β-(1-3)-liées à fixation par une liaison β-(1-6) et sans chaînes β-(1-6)-liées mis à part les chaînes de 4 ou moins de 4 unités de glucose β-(1-6)-liées, ledit glucane pouvant être obtenu par le procédé de mise en contact d'un β-(1-3)-glucane ramifié particulaire insoluble issu de levure ayant des chaînes β-(1-3)-liées et β-(1-6)-liées avec une β-(1-6)-glucanase.

2. Glucane selon la revendication 1, où ledit β-(1-3)-glucane ramifié particulaire insoluble ayant des chaînes β-(1-3)-

liées et β-(1-6)-liées est préparé par le procédé comprenant :

(a) l'extraction alcaline de cellules de levure contenant des glucanes appropriées avec une solution alcaline aqueuse d'extraction appropriée dans des conditions appropriées pour produire un premier résidu de levure insoluble ;
(b) l'extraction alcaline à chaud dudit premier résidu de levure insoluble avec une solution alcaline aqueuse d'extraction appropriée dans des conditions d'extraction appropriées, où l'extraction alcaline à chaud est réalisée au moins deux fois pour produire un second résidu de levure insoluble, et la récupération du résidu de levure insoluble après l'extraction alcaline à chaud ; puis
(c) le lavage dudit second résidu de levure insoluble avec un acide hydrolysant approprié dans des conditions appropriées avec de l'eau à un pH dans le domaine d'environ pH 4 à environ pH 7, pour produire un troisième résidu de levure insoluble, et la récupération dudit troisième résidu de levure insoluble après le lavage ;
(d) l'hydrolyse dudit troisième résidu de levure insoluble dans des conditions d'hydrolyse acide douce, où l'hydrolyse acide est réalisée au moins trois fois pour produire un quatrième résidu de levure insoluble, et la récupération du résidu de levure après chaque hydrolyse acide ; puis
(e) l'ébullition dudit quatrième résidu de levure insoluble dans des conditions appropriées dans l'eau, où l'ébullition dudit quatrième résidu de levure insoluble est réalisée au moins deux fois pour produire un cinquième résidu de levure insoluble, et la récupération du résidu de levure insoluble après chaque ébullition ; et
(f) l'ébullition dudit cinquième résidu de levure insoluble dans des conditions appropriées dans l'éthanol, où l'ébullition dans l'éthanol dudit cinquième résidu de levure est réalisée au moins deux fois pour produire un sixième résidu de levure insoluble, et la récupération du résidu de levure insoluble après chaque ébullition ; puis
(g) le lavage dudit sixième résidu de levure insoluble dans des conditions appropriées avec de l'eau, où le lavage dudit sixième résidu de levure est réalisé au moins deux fois pour produire un glucane de levure, et la récupération du résidu de levure insoluble après chaque lavage.

3. β-(1-3)-glucane solubilisé à chaînes latérales β-(1-3)-liées à fixation par une liaison β-(1-6) et sans chaînes β-(1-6)-liées mis à part les chaînes de 4 ou moins de 4 unités de glucose β-(1-6)-liées, ledit glucane pouvant être obtenu par le procédé de mise en contact d'un produit de glucane ramifié selon la revendication 1 ou la revendication 2 avec un agent solubilisant.

4. Produit de glucane solubilisé selon la revendication 3, où ledit agent solubilisant est l'acide formique et ledit glucane ramifié est mis en contact avec ledit agent solubilisant à une température dans le domaine de 70 à 90°C.

5. Glucane de levure particulaire insoluble, en particulier issu de la famille de levures Saccharomyces, et en particulier de l'espèce de levure saccharomyces cerevisiae, **caractérisé** comme étant un β-(1-3)-glucane ramifié à chaînes latérales β-(1-3)-liées à fixation par une liaison β-(1-6) et sans chaînes β-(1-6)-liées mis à part les chaînes de 4 ou moins de 4 unités de glucose β-(1-6)-liées.

6. β-(1-3)-glucane ramifié de levure de qualité alimentaire à chaînes latérales β-(1-3)-liées à fixation par une liaison β-(1-6) et sans chaînes β-(1-6)-liées mis à part les chaînes de 4 ou moins de 4 unités de glucose β-(1-6)-liées, ledit glucane pouvant être obtenu par le procédé de mise en contact d'un β-(1-3)-glucane ramifié particulaire insoluble de qualité alimentaire issu de levures ayant des chaînes β-(1-3)-liées et β-(1-6)-liées avec une β-(1-6)-glucanase.

7. Glucane de levure de qualité alimentaire selon la revendication 6, où ledit β-(1-3)-glucane ramifié particulaire insoluble de qualité alimentaire ayant des chaînes β-(1-3)-liées et β-(1-6)-liées est préparé par le procédé comprenant:

(a) la mise en contact de parois de cellules de levure avec une solution alcaline aqueuse dans des conditions appropriées pour réaliser l'extraction de protéines et de lipides ;
(b) la séparation des parois de cellules de levure extraites résultantes de ladite solution alcaline aqueuse ;
(c) le lavage des cellules de levure séparées résultantes de manière à en retirer encore des composants des parois de cellules solubilisés ;
(d) la neutralisation des parois de cellules de levure lavées ; et
(e) la pasteurisation des parois de cellules lavées neutralisées puis le séchage des parois de cellules lavées neutralisées pasteurisées résultantes.